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Studies on the Initial Stage of Lipid Oxidation in Bulk Oils.

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STUDIES ON THE INITIAL STAGE OF LIPID OXIDATION IN BULK OILS

Elizabeth Budilarto

This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Afaf Kamal-Eldin

December 2014

Declaration of Original Work

I, Elizabeth Budilarto, the undersigned, a graduate student at the United Arab Emirates University (UAEU) and the author of the dissertation entitled "*Studies on the Initial Stage of Lipid Oxidation in Bulk Oils*", hereby solemnly declare that this dissertation is an original research work done and prepared by me under the supervision of Professor Afaf Kamal-Eldin, in the College of Food and Agriculture at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or similar title at this or any other university. The materials borrowed from other sources and included in this dissertation have been properly cited and acknowledged.

Student's Signature: Lisa B Date: 26 - Jan - 2015

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Abstract (in English)

During the oxidation of bulk oils, oxidation products (i.e. peroxide values, conjugated dienes and thiobarbituric acid reactive substances) are formed gradually and increased sharply at the end of the induction period. Tocopherols were consumed, some water was formed, and micelles increased in size during the induction period of vegetable oils oxidized in bulk. The evidence that the evolution of micellar size was in parallel with the end of induction period corroborates the recognition that micelles are the active site of oxidation. The interactions of α-tocopherol and three synergists: ascorbyl palmitate, phosphatidylcholine and L-lysine were studied in cod liver oil, to examine their effects on formation of thiobarbituric acid reactive substances. Second order polynomial models were found to satisfactorily represent the slope of changes of conjugated dienes, thiobarbituric acid reactive substances and α-tocopherol during the induction period. The suggested optimized levels of the four additives to protect cod liver oil at 30°C based on the rate of thiobarbituric acid reactive substances formation and loss of α-tocopherol (day 0 to 4) are α-tocopherol (1200 μg/g), ascorbyl palmitate (100 μg/g), phosphatidylcholine at (9000 μg/g) and L-lysine (1000 μg/g). Higher level of α-tocopherol and ascorbyl palmitate did not give better protections to the oils or caused a loss in the antioxidant efficacy, compared to when the additives were added at lower levels. Phosphatidylcholine was effective at a wide range of high concentration while L-lysine improved the protection at levels up to 4000 μg/g.

Keywords: induction period, peroxide value, conjugated dienes, thiobarbituric acid reactive substances, water, micelles

Title and Abstract (in Arabic)

دراسات على المرحلة الابتدائية من أكسدة الدهون في الزيوت السائبة

الملخص

اثناء تأكسد الزيوت تتكون بعض منتجات الأكسدة مثل قيمة البيروكسيد، والمركبات ذات الروابط الثنائية المترافقة، لمركبات التي مع حمض الثيوباربيتيوريك. وتزداد كميات منتجات الأكسدة هذه بصورة تدريجية خلال فترة اسة عن اكسدة الزيوت النباتية
السائبة ان مستوى التوكوفيرولات يقل خلال مرحلة الحث وتتكون بعض كميات من المياه و يزداد حجم هذه الحبيبات المستحلبة. وقد أعتبر أن التوازى بين تغير حجم الحبيبات المستحلبة وقيمة البيروكسيد خلال فترة الحث لا في دراسة اخرى تم التركيز
على مقاومة الاكسدة بواسطة مضاد الاكسدة الألفاتوكوفيرول وثلاثة مواد موازرة (هي اسكوربيل البالميتات، جة منوية.
وقد اتضح بناءً على معدلات تكوين المواد المتفاعلة مع حامض الثيوباربيتيوريك خلال اربعة أيام من الأكسدة ان الكميات الأمثل لهذه المضافات لمقاومة الاكسدة فى هذا الزيت هي كالتالي (ميكروغرام/غرام): ألفاتوكوفيرول (1200)، اسكوربيل البالميتات (100)، الفوسفوتيديل كولين (9000)، واللاليسين (1000). كما أنه قد وجد ان اضافة كميات اكثر من التوكوفيرول أو اسكوربيل البالميتات لم تعطي حماية أفضل للزيوت وقد تزيد معدلات الاكسدة وأن الفوسفوتيديل كولين فعالا على نطاق واسع من التركيزات العالية في حين أن ل- لايسين حسن الحماية حتى معدلات تصل إلى 4000 ميكروغرام/غرام.

كلمات البحث: الزيوت السائبة، فترة الحث ، الماء، الحبيبات المستحلبة، مضادات الأكسدة، المركبات الموازرة لمضادات الأكسدة.

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I would like to express my thanks and appreciations to my supervisor, Professor Afaf Kamal-Eldin, who gave me support, guidance, and opportunities to work in the laboratory. She helped me to become independent in research and pursue this Doctoral degree. I would like to extend my thanks to Committee Members in the Department of Food Science and College of Food and Agriculture for their advice, suggestions and support during my study and in my pursuit of a Doctoral degree.

Also, I would like to offer my appreciation to all my friends outside the University for their kindness and continuous support, especially during difficult times.

From my heart, I would like to thank my family, especially my father, mother, and husband who have always supported and encouraged me throughout the years.

Dedication

To my parents and my husband,

who made all of this possible with their encouragement and support.

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Abbreviations

AOT	Bis-(2-ethylhexyl) sulfosuccinate sodium
AP	Ascorbyl palmitate
BDE	Bond dissociation enthalpies
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CD	Conjugated dienes
CMC	Critical micelle concentration
CPP	Critical packing parameter
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DLS	Dynamic light scattering
DOPC	Dioleoylphosphatidyl-choline
DPPC	Dipalmitoylphosphatidyl-choline
DPPE	Dipalmitoylphosphatidyl-ethanolamine
DPPS	Dipalmitoylphosphatidyl-serine
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FFA	Free fatty acid
HLB	Hydrophilic lipophilic balance
IP	Induction or initiation period
LOOH	Lipid hydroperoxide
LOO•	Lipid peroxyl radical

MAG	Monoacylglycerol
MDA	Malondialdehydes
MUFA	Monounsaturated fatty acids
OO	Olive oil
OSI	Oil stability index
o/w	Oil in water emulsion
p-AV	para-Anisidine value
PC	Phosphatidylcholine
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
ROOH	Lipid hydroperoxides
RSM	Response surface methodology
SAXS	Small angle X-ray scattering
SBO	Soybean oil
SDS	Sodium dodecyl sulfate
SFO	Sunflower oil
TAG	Triacylglycerols
TBARS	Thiobarbituric acid reactive substances
TBHQ	*tert*-Butylhydroquinone
α-TOH	α-tocopherol
w/o	Water in oil emulsion

Definitions

Terms	Explanation
Antioxidant effectiveness	The ability of an antioxidant to inhibit the oxidation chain reaction by inactivating radicals during the induction period (Yanishlieva and Marinova, 1992).
Antioxidant strength	The minimum participation of an antioxidant in the side reactions that may increase the rate of oxidation during the induction period (Yanishlieva and Marinova, 1992).
Antioxidant activity	The capability of an antioxidant in bringing the autoxidation chain reaction to an end and to influence the reaction rate during the induction period (Yanishlieva and Marinova, 1992).
Association colloids	Structures or associates that are formed by the surface active components (such as mono- and diacylglycerols and phospholipids) in the presence of small quantities of water in oil media (Chaiyasit *et al.*, 2007).
Co-surfactant	Amphiphiles that improve the solubility of surfactants in the interface and facilitate the formation and stabilization of the micelles (Krei *et al.*, 1995).
Critical micelle concentration (CMC)	The sudden formation of micelles with additional surfactants, which appear as the sharp change of rate of the properties of surfactants when plotted with the concentration of the micelles (IUPAC, 2014).
Cut-off effect of antioxidant effectiveness	A phenomena when polar paradox is not linear (i.e. an increase in hydrophilicity of antioxidant does not cause an increase in the antioxidant effectiveness in bulk oil (Laguerre *et al.*, 2009).
Hydrophilic lipophilic balance (HLB)	A measure of the solubility of a substance (Griffin, 1954).

Interfacial phenomena	In 1994, Frankel proposed that the partition of hydrophilic antioxidant at the air/oil interface of the bulk oil causes them to be effective than the lipophilic antioxidants that are dispersed in the oil phase (Frankel, 1994). Hydrophilic antioxidant is also more soluble in the oils and come in close contact with the ROOH. This was later corrected by the association colloids (see above).
Microenvironment	Aggregates that are formed in bulk oil with the presence of polar and amphiphilic substances and small amount of water and is postulated as the oxidation site in and affects oxidation of bulk oils (Koga and Terao, 1995). This is equivalent to association colloids (see above).
Primary antioxidant	A substance that acts as chain-breaking antioxidants, by scavenging free radicals and converting them to nonradical products (Labuza, 1971).
Secondary antioxidant or synergist	A substance that increases the inhibitory effects of primary antioxidants by regenerating them, scavenging O_2, sequestering metal and/or by other mechanisms which are not completely understood (Eriksson, 1987).
Supramolecular chemistry	"A field of chemistry related to species of greater complexity than molecules, that are held together and organized by means of intermolecular interactions. The objects of supramolecular chemistry are supermolecules and other polymolecular entities that result from the spontaneous association of a large number of components into a specific phase (membranes, vesicles, micelles, solid state structures *etc.*)" (IUPAC, 2014).
Surfactant	Amphiphiles that arrange themselves to form and stabilize the microenvironment (Smit *et al.*, 1991).
Surfactant packing parameter (Sp)	A measure of the ability of a substance to form and stabilize different associates by considering the geometry and curvature preference that are formed by the surfactant (Mitchell and Ninham, 1981).

CHAPTER ONE

INTRODUCTION

Polyunsaturated fatty acids (PUFA) with a high number of double bonds are highly susceptible to oxidation, leading to adverse effects on food flavor, color and shelf life (Drusch *et al.*, 2008) as well as on health and disease (Capitani *et al.*, 2009 and Kathirvel and Rupasinghe, 2011). The major chemical pathways of lipid oxidation have been known for many decades and primarily consist of three stages: initiation, propagation and termination (Labuza, 1971 and Chan, 1987).

Literature describes the initiation phase (also known as induction period (IP), lag phase, or initial stage) as the step where the first lipid hydroperoxides are formed; largely by unknown mechanisms (Privett and Blank, 1962; Labuza, 1971 and Cadenas and Sies, 1998). Catalysts for this reaction are thought to include transition metal ions (Schaich, 2005). The formation of hydroperoxides during the IP is very slow until their concentrations reach a critical value. Then, their rate of formation increases exponentially and the reaction enters the propagation phase (**Figure 1.1**)

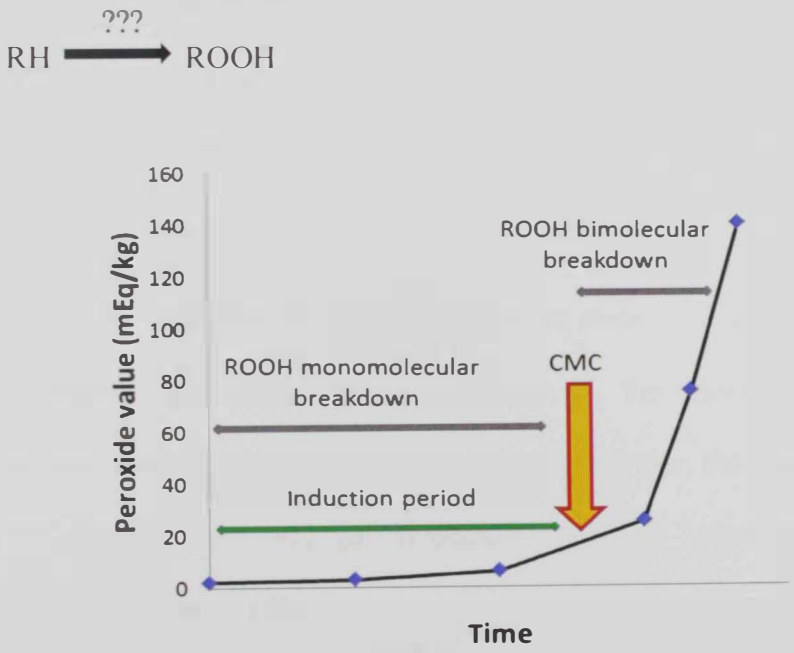


Figure 1.1 Scheme of inhibited lipid oxidation indicating the relationship of the bimolecular period and induction period

Labuza (1971) proposed that during the IP, hydroperoxides are formed and decomposed by monomolecular reactions:

$$ROOH \rightarrow RO\bullet + \bullet OH$$

and at the end of IP, they decompose by bimolecular reactions:

$$2ROOH \rightarrow ROO\bullet + RO\bullet + H_2O.$$

Radicals formed during or at the end of IP may react with each other and terminate the reaction by forming dimers (Schaich, 2005), e.g.:

$$ROO\bullet + ROO\bullet \rightarrow (ROO\text{-}OOR) \rightarrow ROOR + O_2$$

$$RO\bullet + RO\bullet \rightarrow ROOR$$

Miyashita and coworkers (1982a and b) were able to isolate and identify such types of dimers during the early stage of methyl linoleate oxidation.

In the absence of antioxidants, peroxyl radicals (ROO•) propagate chain reactions:

$$ROO\bullet + LH \rightarrow ROOH + L\bullet$$

When antioxidants (AH) are present, the radicals are scavenged by AH and the IP is prolonged (Reische *et al.*, 2002):

$$ROO\bullet + AH \rightarrow ROOH + A\bullet$$

The radicals (A•) generated by the antioxidant are stable and do not carry over the oxidation reaction (Labuza, 1971). During the IP, the antioxidant is gradually consumed and when its concentration reaches a critical value, the reaction reaches the end of IP and enters the propagation phase (Cerne and Lukac-Bajalo, 2006 and Ghaffari and Ghiasvand, 2010).

According to Brimberg (1993a and b) and Ivanova *et al.* (2013), the transition from the initiation to the propagation phase is marked by a critical concentration of hydroperoxides and formation of micelles. Decker and co-workers (2005) suggested

that antioxidants protect lipids against oxidation not only by acting as free radical scavengers but also by modifying reaction sites at the micelles (or association colloids). They proposed that the effectiveness of antioxidants is related not only to their hydrogen donating properties but also their physical locations in the micellar structures. In this case, hydrophilic antioxidants are oriented and partitioned more at the oil/water interface and form micelles in the bulk oils, which do not apply to lipophilic antioxidants (Decker *et al.*, 2005 and Chaiyasit *et al.*, 2008). The evidence that physical factors (micelles) influence oxidation is also observed in emulsion systems, wherein the properties of the emulsion droplets affect the oxidation (Chaiyasit *et al.*, 2007).

Yanishlieva and Marinova (1992) explained that evaluation of antioxidant efficacy should focus not only on the prolongation of the IP but also on the rate of oxidation during the IP. Some antioxidants, such as α-tocopherol, lose efficacy which may witness paradoxical outcomes when very high concentrations are present (Porter, 1980, Porter *et al.*, 1989; Porter, 1993; Huang *et al.*, 1994 and Fuster *et al.*, 1998). The performance of these antioxidants can be improved when synergists are added, e.g. by using a ternary antioxidant system of α-tocopherol, ascorbyl palmitate and phosphatidylcholine in fish oils (Han *et al.*, 1991; Drusch *et al.*, 2008 and Serfert *et al.*, 2009). A synergist is a compound which increases the efficacy or inhibitory effect of primary antioxidants, by lowering the rate of the oxidation reactions (Chan, 1987). The exact mechanisms of synergism are not completely known and require further research. New developments in our understanding of the complexity of lipid oxidation reactions open up new possibilities for explanatory and applied research in this field.

1.1 The objectives of the present dissertation

This dissertation focuses on the initiation period and the transition from initiation to propagation period. The specific aims were to

1. Investigate water formation and micellization and their relations to peroxide buildup during the oxidations of bulk vegetable oils (**Chapter 3**).
2. Investigate the synergisms between α-tocopherol and three synergists (ascorbyl palmitate, phosphatidylcholine and L-lysine) in retarding the oxidation of cod liver oil in bulk (**Chapter 4**).

CHAPTER TWO

LITERATURE REVIEW

The Supramolecular Chemistry of Lipid Oxidation and Antioxidation in Bulk Oils

2.1 Introduction

Despite lipid's important functions as food components, contributing to nutritive values and functionality (Bandarra *et al.*, 1999; Jacobsen *et al.*, 2008 and Shahidi and Zhong, 2010), lipids are susceptible to autooxidation. Polyunsaturated fatty acids, such as omega-3 fatty acids of fish lipids, undergo fast oxidation, which deteriorates the lipids sensory quality, and hence limits their shelf-life (Schaich, 2005; Choe and Min, 2006; Pinedo *et al.*, 2007; Chen *et al.*, 2010 and Chen *et al.*, 2011a). Many attempts have taken place in order to protect unsaturated fatty acids from autoxidation however, no complete successful (or satisfying) solutions have been found to protect lipid products (Kamal-Eldin *et al.*, 2003).

The chemistry of lipid oxidation has been studied for decades, and it is known to occur in three phases: initiation or induction (IP), propagation and termination (Kamal-Eldin *et al.*, 2003; Schaich, 2005; and Shahidi and Chandrasekara, 2010). The theory of autocatalysis of lipid oxidation by hydroperoxides (LOOH) was developed in the 1940's (Farmer *et al.*, 1943). However it is still applicable today, due to its satisfactory explanation of the chemical reactions which occur during the oxidation of lipids in the three phases mentioned above (Velasco and Dobarganes, 2002; Yanishlieva *et al.*, 2002; Choe and Min, 2006; and Chaiyasit *et al.*, 2007). In addition, the formation of volatile oxidation products deteriorates the flavor and odor of the lipids (Choe and Min, 2006; Tabee *et al.*, 2008; Laguerre *et al.*, 2009; and Shahidi and Zhong, 2010). This hydroperoxide theory that was proposed by Farmer and colleagues (Farmer *et al.*, 1943) gives a general description of the role of antioxidants in retarding lipid autoxidation, however it is frequently imprecise, inconsistent and fails to explain paradoxical outcomes of certain studies (Kamal-Eldin and Appelqvist, 1996; Bandarra *et al.*, 1999; Wanasundara and Shahidi, 2005; Shahidi and Zhong, 2010; and Chen *et al.*, 2011b). Since there is evidence which suggests the presence of physical factors

that could influence lipid oxidation, and because bulk oils are not homogenous, research has been going on for two decades, to seek the explanation of the other aspects of lipid oxidation that cannot be merely explained by chemical reactions. These studies for instance include investigating molecular positions in space, particularly at the interfaces of nanoemulsions in bulk oils. Bulk oils are considered as water-in-oil nanoemulsions rather than pure lipid phases. The objective of this study is therefore to review the present knowledge of lipid oxidation and focus on the physical factors that affect the oxidation of bulk oils, and examine also the effects of antioxidants in relation to these physical factors.

2.2 Antioxidants and Their Mechanisms

The general definition of an antioxidant is "Any substance that, when present at low concentrations compared with those of an oxidizable substrate, delays or prevents the oxidation of that substrate" (Halliwell, 1990, page 1). According to their mechanisms in protecting the lipids, antioxidants are classified into primary and secondary antioxidants (McClements and Decker, 2000; Choe and Min, 2006; and Chen *et al.*, 2011b).

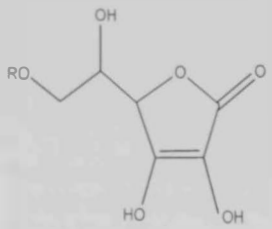
(1) Primary antioxidants are those that act as chain-breaking antioxidants. These antioxidants are able to react readily with free radicals (accept free radicals with more affinities to RO_2• than fatty acyls), such as lipid, alkoxyl and peroxyl radicals and convert them to more stable, low energy, nonradical products, so called antioxidant radicals (A•) (McClements and Decker, 2000; Wanasundara and Shahidi, 2005; and Sun *et al.*, 2011). These classes of antioxidants also scavenge oxygen. They prolong the IP and disrupt the propagation stage (McClements and Decker, 2000 and Shahidi and Zhong, 2010). Primary antioxidants are mostly

phenolic compounds, mono- or polyhydroxy phenols with hydrogen-donating substitutions on the ring (Wanasundara and Shahidi, 2005 and Choe and Min, 2006), such as BHA, BHT, TBHQ, tocopherols, and flavonoids. Structures of antioxidants discussed in this Chapter are drawn in **Figure 2.1**. The antioxidant potency of these phenolic compounds is influenced by their hydrogen donation ability (i.e. related to the number of O-H groups in *ortho* and *para* positions), whether these phenolic hydrogens are hydrogen bonded and their bond dissociation enthalphies (BDE) (Wanasundara and Shahidi, 1998; Schaich, 2005; and Chaiyasit *et al.*, 2007). Other mechanisms may also be attributed to the antioxidant activity of primary antioxidants, besides as free radicals scavengers (Nenadis *et al.*, 2003), such as as oxygen sequesters, metal chelations and have a role in the light energy absorption. Examples of multiple-function antioxidants are propyl gallate, proanthocyanidins and ascorbic acid (Chaiyasit *et al.*, 2007).

(2) Secondary antioxidants are also called retarders. These antioxidants increase the efficacy of the inhibitory effect of primary antioxidants, and are also called synergists. This class of antioxidants includes sequestrants or chelating agents (e.g. phytic acid, EDTA and citric acid), oxygen scavengers and reducing agents (e.g. ascorbates) and other effects that are not completely understood (e.g. amino acids and phospholipids) (Wanasundara and Shahidi, 2005 and Choe and Min, 2006). The exact mechanisms of action of the wide variety of secondary antioxidants have not been properly understood but some of their speculated activities include chelating prooxidants or catalysts, providing hydrogen to primary antioxidants, decomposing LOOH to nonradical species, scavenging ground state and singlet oxygens, and absorbing UV light (McClements and Decker, 2000). Brimberg (Brimberg, 1993a and b) proposed a theory, that another mechanism by which

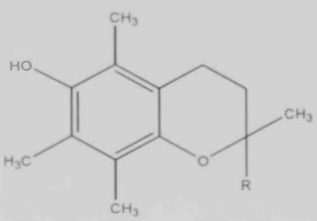
secondary antioxidants contribute to the protection effects in bulk oils is due to their stabilization or micellization effects (arrangement in microenvironments). Unfortunately, this theory was not realized until some years later.

Ascorbic acid, ascorbyl palmitate



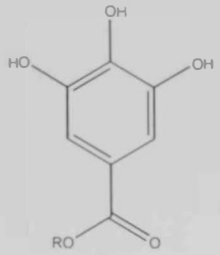
Ascorbic acid, R = H;
Ascorbyl palmitate, R = $CH_3(CH_2)_{13}CO$-

Trolox and α-tocopherol



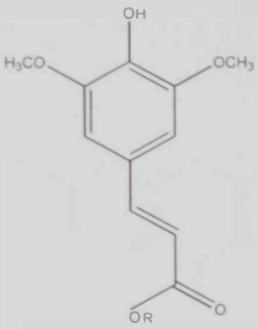
Alpha tocopherol, R = $C_{16}H_{33}$; Trolox, R = -COOH

Gallic acid, octyl gallate



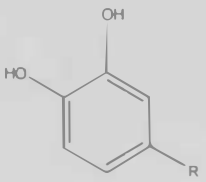
Gallic acid, R = H;
Octyl gallate, R = $-(CH_2)_7-CH_3$

Cinnamic acid derivatives



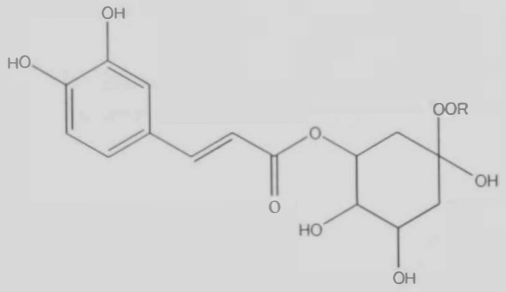
Sinapic acid, R = H;
Sinapine, R = $-(CH_2)_3N(CH_3)_3$

Caffeic acid and its derivatives



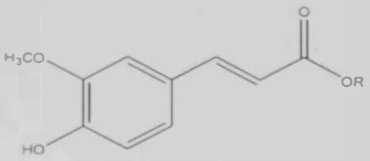
Caffeic acid, R = $-CH_2=CH_2-COOR_1$ where R_1 = H;
Caffeic acid phenethyl ester, R = $-CH_2=CH_2-COOR_1$ where R_1 = $-CH_2-CH_2$-Phenol;
Dihydrocaffeic acid, R = $-CH_2-CH_2-COOH$;
Propyl caffeate, R = $-CH=CH_2-COO-(CH_2)_2-CH_3$;
Propyl hydrocaffeate, R = $-CH_2-CH_2-COO-(CH_2)_2-CH_3$

Chlorogenic acid and its derivatives



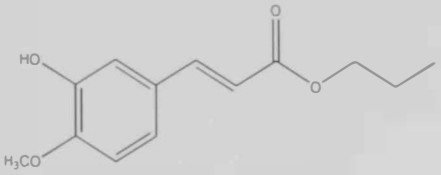
Chlorogenic acid, R = H
Chlorogenic acid esters:
Butyl chlorogenate, R = $-(CH_2)_3-CH_3$
Dodecyl chlorogenate, R = $-(CH_2)_{11}-CH_3$
Hexadecyl chlorogenate, R = $-(CH_2)_{15}-CH_3$

Ferulic acid and derivatives

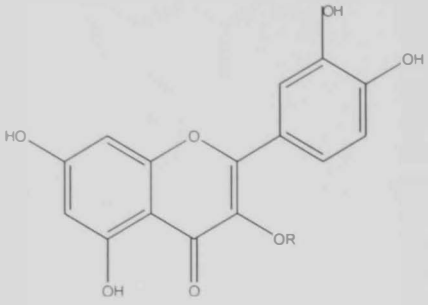


Ferulic acid, R = H;
Ferulic acid phenetyl ester, R = $-(CH_2)_3$-Ph;
Alkyl ferulates:
1-pentyl ferulate, R = $-(CH_2)_4-CH_3$
1-hexyl ferulate, R = $-(CH_2)_5-CH_3$
1-heptyl ferulate, R = $-(CH_2)_6-CH_3$
Propyl ferulate, R = $-(CH_2)_3-CH_3$

Propyl isoferulate

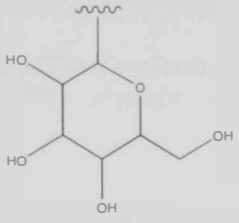


Quercetin and its derivatives

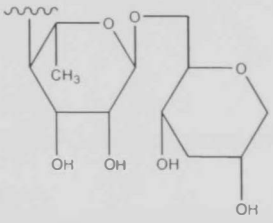


Quercetin, R = H

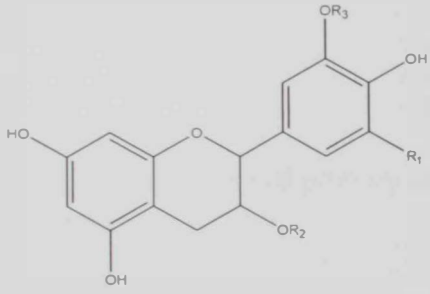
Quercetin-3-O-glucoside, R=



Rutin, R =

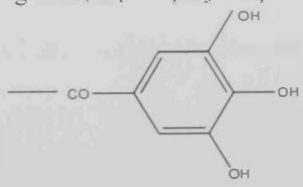


Epi-catechins and derivatives



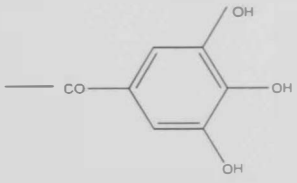
Epicatechin, R_1 = H, R_2 = H, R_3 = H
Epicatechin gallate, R_1 = H, R_3 = H,

R_2 =



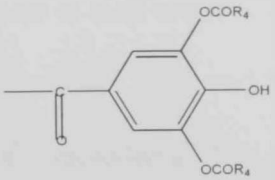
Epigallocatechin, R_1 = OH, R_2 = H, R_3 = H
Epigallocatechin gallate (EGCG), R_1 = OH, R_3 = H,

R_2 =

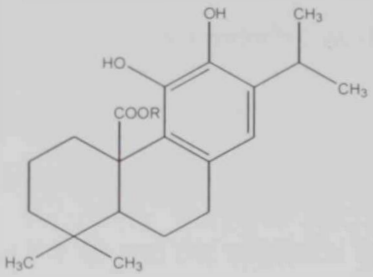


EGCG ester (stearate), R_1 = $OCOR_4$, R_3 = $OCOR_4$,
where R_4 = $(CH_2)_{16}CH_3$

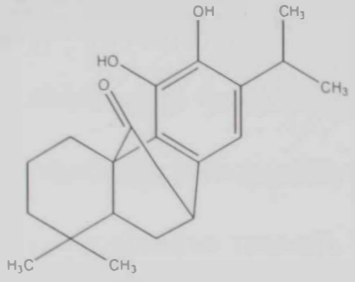
R_2 =



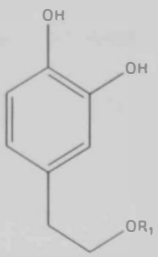
Carnosic acid and derivatives



Carnosic acid, R = H
Methyl carnosate, R = CH_3
Carnosol

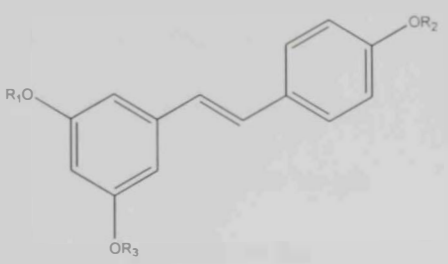


Hydroxytyrosol



Hydroxytyrosol, R_1 = H;
Hydroxytyrosol acetate, $R_1 = COR_2$, where R_2 = -CH_3;
Hydroxytyrosol butyrate, $R_1 = COR_2$, where R_2 = -$(CH_2)_2$-CH_3;
Hydroxytyrosol laurate, $R_1 = COR_2$, where R_2 = -$(CH_2)_{10}$-CH_3;
Hydroxytyrosol palmitate, $R_1 = COR_2$, where R_2 = -$(CH_2)_{14}$-CH_3;
Hydroxytyrosol stearate, $R_1 = COR_2$, where R_2 = -$(CH_2)_{16}$-CH_3;
Hydroxytyrosol oleate, $R_1 = COR_2$, where R_2 = -$(CH_2)_7$-CH=CH-$(CH_2)_7$-CH_3;
Hydroxytyrosol linoleate, $R_1 = COR_2$, where R_2 = -$(CH_2)_7$-CH=CH- CH_2-CH=CH-$(CH_2)_4$-CH_3;
Hydroxytyrosol octanoate, $R_1 = COR_2$, where R_2 = -$(CH_2)_6$-CH_3

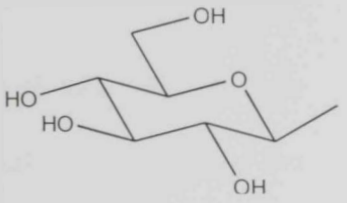
Resveratrol



Resveratrol, R_1 = H, R_2 = H, R_3 = H
Acylated resveratrols:
3-stearoylresveratrol, R_1 = -$CO(CH_2)_{16}CH_3$, R_2 = H, R_3 = H
4'-stearoylresveratrol, R_1 = H, R_2 = -$CO(CH_2)_{16}CH_3$, R_3 = H

Glucosylated resveratrols, has the same backbone structure as resveratrol but with

R_1 =



Resveratrol-3-β-glucopyranoside, R_2 = H
R_3 = H;
Resveratrol-3,5-di-β-glucopyranoside,
R_2 = H
R_3 = β-glucose;
Resveratrol-3,4'-di-β- glucopyranoside,
R_2 = β-glucose, R_3= H

Figure 2.1. Structures of primary antioxidants with different polarity discussed in Chapter 2

To have better protection, combinations of primary and secondary antioxidants are frequently found to provide more efficiency than the sum of their single actions in retarding lipid autoxidation (Hamilton *et al.*, 1998; Bandarra *et al.*, 1999; and Shahidi and Zhong, 2010). Synergisms between the two types of antioxidants often lead to prolonged IP and/or decrease the reaction rate during IP (Yanishlieva *et al.*, 2002 and

Yanishlieva and Marinova, 2003). Examples of synergistic effects are between tocopherols and ascorbic acid, and between the mixtures of natural tocopherols and citric acid (Wanasundara and Shahidi, 2005). There are three descriptive parameters to evaluate the primary and secondary antioxidant's efficacy, by considering the length of the IP and the oxidation rate during this IP, as proposed by Yanishlieva and co-workers (Yanishlieva and Marinova, 1992 and references cited therein):

1. Effectiveness, which is the ability of the antioxidants inhibiting the radical chain process by inactivating $RO_2\bullet$ during the IP. Effectiveness can be measured by the stabilization factor (F) which is IP_{inh} divided by IP_o, wherein IP_{inh} is the IP of a retarded oxidation (with an antioxidant), and IP_o is the IP of the unretarded oxidation (no antioxidant present).
2. Strength is defined as the measure of the antioxidant to contribute to side reactions which can change the rate of oxidation during IP. Strength is measured by the oxidation rate ratio, ORR that is W_{inh} / W_o. W_{inh} is the rate of oxidation with an antioxidant and W_o is the rate of oxidation without an antioxidant. When the addition of the antioxidant causes faster oxidation rates than if it is without the antioxidant, then the ORR value will be more than 1.
3. Antioxidant activity (A) measures two things, the effectiveness of an antioxidant in bringing the autoxidation chain to an end and the antioxidant's capability to influence the reaction rate during IP. Antioxidant activity can be calculated by F / ORR (Yanishlieva *et al.*, 2002 and Yanishlieva and Marinova, 2003).

Most the previous studies observed synergistic interactions between primary and secondary antioxidants which explained or assumed that their contributions to inhibit oxidations are due to their possible chemical interferences. In this case, primary antioxidants and synergists often prolong the IP and lower reaction rates during the IP

(W_{inh}). An example of this is presented in **Figure 2.2**: the inhibition of autoxidation of fish oil at 20°C by ascorbyl palmitate (1000 ppm) and lecithin (5 ppm) (Hamilton *et al.*, 1998). Other examples include the inhibition of oxidations of fish oil at 20°C with ascorbyl palmitate (500 ppm) and lecithin (2000 ppm) (Drusch *et al.*, 2008), soybean oil at 110° C with α-tocopherol (4000 ppm) and phospholipids (15,000 ppm) (Hildebrand *et al.*, 1984), and peanut oil at 110° C with α-tocopherol (1000 ppm) and phospholipids (1500 ppm) (Chu and Hsu, 1999). Indigenous minor components in refined bulk oils such as phospholipids and monoacylglycerols can also change the length of the IP and the rate oxidation by acting as synergists to tocopherols (in the case of phospholipids) or antagonists (such as monoacylglycerols) (Chaiyasit *et al.*, 2007 and 2008; and Chen *et al.*, 2011a and b and 2012a and b). Besides synergisms, there are more situations with unexplained phenomena which influence the reaction rates of oxidation when antioxidants are present. One classical case is the loss of antioxidant activity when primary antioxidant concentrations are elevated, such is the case with α-tocopherol (Fuster *et al.*, 1998 and Yanishlieva and Marinova, 2003). The concept of *side reactions* was introduced to explain such paradoxical outcomes with regards to the loss of efficacy at higher levels of antioxidants (Fuster *et al.*, 1998).

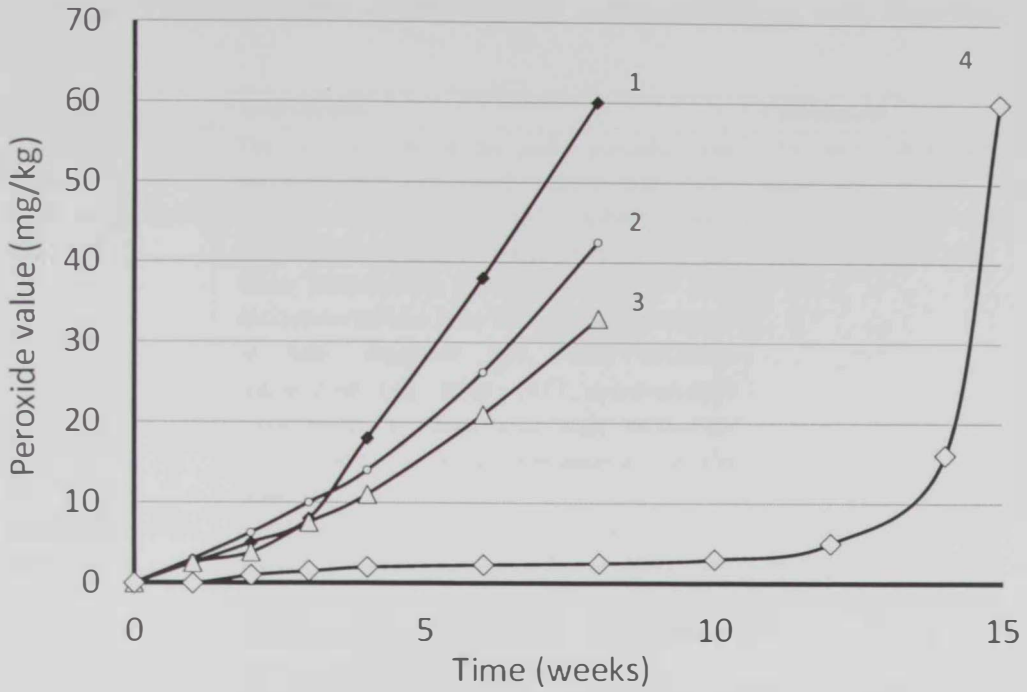


Figure 2.2. Synergisms of ascorbyl palmitate and lecithin on the autoxidation of fish oil at 20°C; (1) no antioxidant, (2) 0.1% ascorbyl palmitate, (3) 0.5% lecithin, (4) 0.1% ascorbyl palmitate + 0.5% lecithin. Data from Hamilton *et al.* (1998).

2.3. The antioxidants polar paradox and interfacial phenomena

The previous sections explained that a pure chemical model was insufficient to explain the effects of antioxidants (primary and secondary), in terms of their synergistic interactions on the rates of lipid oxidation reactions. In addition, researchers are continuing to investigate the other aspects of oxidation and antioxidants and have strong results that indicate the presence and effects of different molecular species which self-assemble and orient themselves in bulk oils. The historical development of the roles of physical assemblies of lipid soluble components on lipid oxidation is presented in **Table 2.1**. This knowledge helps to elucidate the phenomena and paradoxical outcomes that are not yet well understood.

Table 2.1. Physical effects of additives on lipid oxidation in bulk oils: historical account

References	Observations	Conclusions
Porter, 1980; Porter *et al.*, 1989; and Porter, 1993	The general rule of the *polar paradox* was proposed and confirmed stating that polar antioxidants (e.g. propyl gallate, *tert*-Butylhydroquinone (TBHQ), Trolox C) are more effective in food systems with low surface-to-volume ratio or nonpolar lipids such as bulk vegetable oils while nonpolar antioxidants (e.g. BHA, BHT, α-tocopherol) work better in foods with high surface-to-volume ratio or polar lipid emulsion such as o/w emulsion.	The antioxidant activity is oppositely related to the polarity of antioxidants in relation to food lipids.
Brimberg, 1993a and b	Lipid hydroperoxides (LOOH) are surface-active agents that form micelles at above their critical micelle concentration (CMC). O_2 is maximumly solubilized in lipids when hydroperoxide CMC is attained.	Micelles formed by hydroperoxides are the site of lipid oxidation reaction.
Frankel *et al.*, 1994 and 1996a and b	Lipophilic antioxidants (e.g. α-tocopherol and ascorbyl palmitate) were more effective in o/w emulsion system than in bulk oil because they had more affinities toward water-oil interface, while the opposite was true for hydrophilic antioxidants (Trolox, ascorbic acid, rosmarinic acid, carnosic acid, rosemary extract), which were more oriented in air-oil interfaces in bulk oil. Mixtures of α-tocopherol and ascorbic acid were more active in bulk oils than in o/w emulsions.	The *interfacial phenomenon* was used to explain the polar paradox. This phenomenon is related to the kinds of interfaces at which the antioxidants are more oriented, which may explain the polar paradox.
Koga and Terao, 1995	In aqueous microenvironments in bulk lipids (15:85 by mol/mol mixture of methyl linoleate and methyl laurate), phospholipid aggregates enhanced the accessibility of α-tocopherol to radicals and hence the interruption of chain initiation. The polar OH group of α-tocopherol is located not too deeply in hydrophobic region of phospholipid bilayer membrane but just near by the membrane surface.	Interfacial microenvironment is the place where interactions among surfactants, antioxidants and radicals take place.
Huang *et al.*, 1996a	Linoleic acid competed with Trolox for Tween 20 in the polar region of the micelles and at the o/w interface. Trolox diffused in the water phase and the mixed micelles and thus was a better antioxidant than α-tocopherol that was diffused in the oil phase.	Micelle is where the oxidation and interactions of antioxidants and surfactants take place.
Carlotti *et al.*, 1997	An emulsion was known to contain micellar structure. L-tryptophan was a very effective synergist with α-tocopherol because it was distributed in the micellar core or in the o/w interface.	Micelle core and interface have different roles in autoxidation.

Endo *et al.*, 1997a, b and c	A mixture of trieicosapentaenoylglycerol and tripalmitoylglycerol (2:1, mole/mole) was most susceptible to oxidation than other ratios. The triacylglycerol (TAG) structure affected the oxidation rate of unsaturated fatty acids. TAGs with unsaturated fatty acids at sn-2 positions were more stable than those having unsaturated fatty acids at sn-1 and sn-3.	Physical structures, such as the position of fatty acids on TAG, have an effect on lipid oxidation.
Hamilton *et al.*, 1998	Lecithin solubilizes ascorbyl palmitate and enhances its physical interactions with α-tocopherol which form reversed micelles. This versatile network had an ability to interrupt free-radical propagation by inhibiting the participation of ascorbyl radical in promoting LOOH scission.	Reversed micelles are formed in w/o emulsions.
Frankel and Meyer, 2000	The effectiveness of antioxidants in a system is influenced by several factors including the partitioning behavior of antioxidants between lipid and aqueous phase, the oxidation conditions and the physical state of the oxidizable substrate. Surface-active substances influence the interfacial interactions between the system and antioxidant. The oil-water partition coefficients influence the distribution of relatively polar antioxidants in the lipid and aqueous phase of a food emulsion. Trolox, which is very polar, works very well in bulk oil and is more effective in o/w emulsions of linoleic acid compared to those of TAG. Unlike TAG, linoleic acid is more polar and forms micelles in aqueous systems. Micelle-forming substrates enhance the activity of hydrophilic and polar antioxidants.	o/w partition coefficient can explain the affinity of a compound in a lipid and aqueous phase.
Khan and Shahidi, 2000	The synergistic interactions of tocopherols and phospholipids in borage and evening primrose TAG can be explained partly by phosphatidylcholine increasing the accessibility of α-tocopherol in the aqueous microenvironment where the induction of lipid oxidation occurs.	Phospholipids act as synergists and support antioxidants by modifying the reaction environment.
Schwarz *et al.*, 2000	Antioxidants (Trolox, propyl gallate, gallic acid, methyl carnosoate, carnosic acid) have moderate or higher activity in bulk oils than in emulsions. Polar antioxidants (propyl gallate & gallic acid) exhibit prooxidant or no antioxidant activity in polar media (o/w emulsions). Emulsifiers (Cetheareth-15, glyceryl stearate, polyglyceryl glucose methyl distearate) form lamellar structures in bulk oils & nonpolar media causing higher solubilization and activity of polar antioxidants.	The activity of antioxidants can be enhanced or reduced by emulsifiers. Mesophase structures depend on molecular structure and critical packing parameter (CPP) of the compound.

Gupta *et al.*, 2001	Inverse micellar structures (~ 60 Å in diameter) were formed by phospholipids in a hexane-oil mixtures containing < 0.3% water. The principal domains of the phase behavior include micellar solution, two phase dispersion, and dense micellar solution. A smooth transition to dense micellar phase was observed with increased phospholipids concentration. Dynamic light scattering measurements showed that aggregate sizes were affected by the amount of phospholipids and >1.5% water, below which the available water is very limited to significantly affect core sizes.	Reversed micelles are formed in w/o nanoemulsions. The size of aggregates depends on the amount of surfactants and water.
Kortenska *et al.*, 2001	Polar products of lipid oxidation with oxygen containing groups (e.g. LOOH, fatty alcohols, acids, water) tend to associate in non-polar media to form aggregates. Fatty alcohols may play a role as an initiation on the aggregate formation and hence influence lipid oxidation.	Polar products of lipid oxidation affect the oxidation rate by modulating the reaction environment.
Kortenska *et al.*, 2002	High concentrations of polar compounds (e.g. LOOH, lipid peroxyl radical, and BHT form microaggregates in the presence of fatty alcohols. This leads to an increase of the rate of termination and causes a decrease in the efficiency of BHT to protect purified sunflower oil (SFO) since LOOH decompose faster inside the polar interior of the micro aggregate.	Fatty alcohols or BHT might act as surfactants and form microaggregates (micelles) in the w/o system.
Velasco and Dobarganes, 2002	Cloudy olive oil (OO) was more oxidatively stable than filtered OO. Suspended and dispersed materials in cloudy OO play a stabilization role by acting as antioxidants and/or as a buffer and preventing acidity increases.	Polar constituents in oils, e.g. unsaponifiable materials, may play a physical role in oil solubilization.
Brimberg and Kamal-Eldin, 2003a	LOOH formed during methyl linoleate oxidation are surface-active and can form micelles. When LOOH concentration reaches CMC, lipid oxidation enters the propagation period.	CMC of hydroperoxides marks the beginning of propagation period.
Brimberg and Kamal-Eldin, 2003b	The amount of oxygen solubilized in lipid is comparative to the number of micelles formed during oxidation. When lipid medium has conjugated double bonds which are oxidized, no hydroperoxides are formed. Instead cyclic peroxides that are not surface-active and do not form micelles are formed, hence there is no propagation period.	Organic peroxides (not hydroperoxides) are not surface active and do not atfect the oxidation rate.
El-Shattory *et al.*, 2003	Reversed micelles were formed with surfactant aggregates in organic solvents, e.g. LOOH, methylglucose dioleate, polyglyceryl-3-oleate, lecithin.	Reversed micelles are formed in organic system in the presence of surfactants.

Kiokias and Gordon, 2003	The activity of norbixin as an antioxidant in bulk oil is consistent with the polar paradox. Norbixin is soluble in water (as aggregates) and oriented at the oil-water interface in the emulsion due to its massive hydrocarbon backbone but it is insoluble in oil.	Norbixin is an example to supports the polar paradox.
Decker *et al.*, 2005	Differences in the effectiveness of the antioxidants in oil systems are mainly due to their physical location in the system (the antioxidant paradox). Polar antioxidants are more effective in bulk oil because they can accumulate at the air-oil interface or in reversed micelles within the oil, where lipid oxidation occurs. On the other hand, nonpolar antioxidants are more effective in o/w emulsions because they accumulate in the oil droplets and/or may accumulate at the oil-water interface, where interactions between LOOH at the droplet surface and pro-oxidants (e.g. transition metals) take place.	Antioxidants effectiveness depends on how and where the antioxidants are partitioned in the system. In bulk oil, lipid oxidation occurs at the air-oil interface as well as in the reversed micelle (oil-water) interface.
Calligaris and Nicoli, 2006	Salts with the antichaotropic anionic species were able to form weak bonds. These may form a "hydrophilic" structure around them and inhibit the solubility of other substances with lower polarities. Thus, these salts may enhance the activity of certain antioxidants.	Hydrophobic structure formed by the salts might salt-out amphiphilic molecules and affect lipid oxidation.
Becker *et al.*, 2007	Antioxidant activity in bulk oil was related to the polarity of the antioxidants, within the order: quercetin > α-tocopherol >> astaxanthin = rutin. Rutin was an exception in that it is relatively hydrophilic but had the lowest activity in bulk oil. This indicated that it is not only the polarity that govern the effectiveness of antioxidants. Poor solubility of rutin in bulk oil or degradation of its glycoside at high temperature also influenced its effects.	Hydrophilicity (or lipophilicity) do not always correlate with the antioxidant effectiveness in bulk oil.
Chaiyasit *et al.*, 2007	Edible oils contain polar lipids (e.g. monoacylglycerol (MAG), diacylglycerol (DAG), free fatty acid (FFA), phospholipids, sterols, cholesterols, phenolic compounds, aldehydes and ketones), which have amphiphilic nature. Components with especially low HLB can self-assemble due to hydrophobic interactions and form association colloids, including lamellar structures and reversed micelles. These surface active molecules partition at the o/w interface and induce the concentration of antioxidants at the surface of colloids, thus increasing interactions between antioxidants and/or prooxidants with metal at the interface or water core.	The term *association colloids* was proposed for geometric forms such as lamellar structures and reversed micelles, which are formed by surfactants.

Chaiyasit *et al.*, 2008	Edible oils contain surface-active compounds and water that can form physical structures such as reversed micelles. Both phosphatidylcholine and oleic acid were suggested to be located at the o/w interface by 5-dodecanoylaminofluorescein probe measurement, and phosphatidylcholine was found to increase the accessibility of α-tocopherol to radicals while oleic acid acted as prooxidants.	More examples of the effects of surface-active compounds and reversed micelles on lipid oxidation were presented.
Kasaikina *et al.*, 2008	LOOH do not form classical micelles but form associates (1-500 nm in size) alongside water, surfactants, alcohols, acids, ketones and other ox. products. LOOH is amphiphilic and concentrates on the boundary of micelle and water. In a natural olefin (limonene), cationic surfactant promotes oxidation, whereas anionic and nonionic surfactants did not have any influence.	Association colloids rather than micelles were advocated. Charges of surfactants affect the role of the surfactants as an antioxidant or prooxidant.
Koprivnjak *et al.*, 2008	Bipolar molecules such as lecithin form reversed micelle where their polar groups are pointed toward the interior and their nonpolar tails are directed toward the exterior (oil). Lecithin ability to increase oxidative stability was due to its bipolar character and its ability to entrap hydrophilic antioxidants to concentrate on the micellar interface.	Phospholipids stabilize reversed micelles.
Laguerre *et al.*, 2009	Not all nonpolar antioxidants behave as antioxidant in polar medium; the antioxidant capacity of homologous series of chlorogenic acid esters in o/w emulsions increased as the alkyl chain length increased until dodecyl chain. Further chain extension caused a drastic drop of antioxidant capacity (a cut-off effect).	The Polar Paradox is not linear. As the alkyl chain length increases, the hydrophilicity and the antioxidant activity in o/w emulsions increase to a certain extent, but a further increase reduces the antioxidant activity (a cut-off effect).
Belhaj *et al.*, 2010	The size of nanoemulsions was influenced by the pressure, oil composition, and the surface-active properties of surfactants. Changes of α-tocopherol antioxidative effect in bulk oil was more significant than that in emulsions.	The importance of nanoemulsions in lipid oxidation was advocated.
Bendini *et al.*, 2009	When virgin OO was subjected to temperature close to 0°C, changes in the physical state happened leading to destabilization of the microdroplets of water and the concentration of polar phenolic compounds and finally the loss of antioxidant activity.	At lower temperatures (~0 °C), destabilized microdroplets in bulk oils may accelerate the rate of lipid oxidation.

Chen *et al.*, 2010	When the phospholipid concentration exceeds their CMC, reversed micelles were formed. Dioleoylphosphatidylcholine and water formed spherical association colloids in SBO, and they were prooxidative because more (small) non-scattering association colloids were formed. 1,2-dibutyryl-*sn*-glycero-3-phoshocholine formed cylindrical structures and had no impact on oxidation rates.	As amount of surfactant increased, CMC was affected and so the formation of reversed micelle. The kinds of physical structures differently affect oxidation. Spherical shapes of association colloids were prooxidants, while cylindrical shapes had no impact on oxidation rates.
Granıza-Michalowska and Stachowiak, 2010	Astaxanthin causes no protection of bulk oils, which indicates that antioxidant activity was correlated with its polarity. Astaxanthin is hydrophobic, it is located in the oil not at the air-oil interface protecting o/w emulsions, but not in bulk oils and liposome.	Lipophilic compounds do not affect the oxidation in bulk oils.
Kasaikina *et al.*, 2010	Primary amphiphilic products of the oxidation of LOOH and lipids, and cationic surfactants form mixed micelles, which accelerated the decomposition of LOOH and other polar components (e.g. metal-containing compounds, inhibitors etc.)	Mixed micelles with different geometric forms were detected in w/o emulsions that enhance the decomposition of LOOH.
Medina *et al.*, 2010	The effectiveness of antioxidants relies on its chemical reactivity (as radical scavenger or metal chelator), its interaction with other food components, their concentration and physical location in homogeneous or heterogeneous system. For instance, resveratrol had a low activity in inhibiting lipid autoxidation in w/o emulsions and bulk oil because it has a low incorporation in the droplet interface and its poor solubilty in water, thus it is probably located far away from the air-oil interface.	The importance of physical effects of antioxidants was highlighted.
Chen *et al.*, 2010	Amphiphilic surface active compounds, which exist after oil refining (such as MAG, DAG, phospholipids, sterol, and FFA), interact with water to form association colloids (reversed micelles, microemulsions, lamella and cylindrical aggregates). Increasing water concentration had very little impact on the IP of lipid oxidation (by hexanal) at 55°C. MAG formed ordered lamellar structures in hazelnut oil. Association colloids impact on lipid oxidation depends on the additives ability to form the colloids and how the additives are partitioned in the micelles.	Different surfactants form different kinds of mesophase structures that affect lipid oxidation. Water concentration had a limited effect on oxidation at 55°C.
Chen *et al.*, 2011b	Lipid oxidation is not only influenced by the traditional chemical factors, such as lipid compositions, transition metals but also by the existence of physical structures. Phospholipids form microstructures known as association colloids within soybean oil (SBO). Reversed	Physical structures are important affectors of lipid oxidation.

	micelle of dioleoylphosphatidylcholine shorthened the IP of SBO at 55°C.	
An *et al.*, 2011	Antioxidative and prooxidative properties are determined by internal factors (i.e. the oxidation substrates, structural organization and the microenvironment for the bioactive compound) and external factors (i.e. heat, pressure and exposure to light). Hydrophobic alkyl chain increased water insolubility of 7-n-alkoxydaidzeins: daidzein, 7-n-butyloxy-daidzein, 7-n-octyloxy-daidzein, 7-n-dodecyloxy-daidzein and 7-n-hexadecyloxy-daidzein. Daidzein increased membrane fluidity, but 7-n-butyloxy-daidzein until 7-n-hexadecyloxy-daidzein decreased fluidity. The compounds appeared to be present in the central domain of the liposome bilayer in the order 7-n-dodecyloxy-daidzein > 7-n-octyloxy-daidzein > 7-n-butyloxy-daidzein > 7-n-hexadecyloxy-daidzein > daidzein, leaving 7-n-dodecyloxy-daidzein as the most effective antioxidant, monitored by a fluoresence spectroscopy with a fluorescence probe.	Changes in the hydrophilicity of an antioxidant affect its inhibitory activity of lipid oxidation.
Shahidi and Zhong, 2011	The distribution of polar antioxidants at the oil-air interface was examined because air is much less polar than oil. Antioxidants action was influenced by various environments (such as lamellar and reversed micelles) which are formed by water, amphiphilic compounds (e.g. LOOH, aldehydes and ketones) which alter the physical location of antioxidants. The association colloids are the site of lipid oxidation in bulk oil. A cutoff effect was observed and occurred wherein antioxidant activity increases as the alkyl chain lengthens until a threshold is achieved. Then a further increase of the chain length caused a drastic decrease of activity. Molecular size also influenced antioxidant effectiveness, antioxidants with bulky structures (e.g. phenolic derivatives with long alkyl chains). It also had a steric hindrance and thus lower mobility than those of smaller size, therefore lowered diffusibility toward reactive centers.	A cut-off effect was found for hydrophilic antioxidant in nonpolar medium.
Sorensen *et al.*, 2011	w/o emulsion resembles bulk oil, of which water is located in micelles and is surrounded by emulsifier. The efficacy of antioxidants in emulsions of water in omega-3 lipids follow the polar paradox, but not for the o/w emulsion. In w/o, at pH 7, ascorbic acid had negative charges and repulsive forces existed between the interface and ascorbic acid, thus it was located	w/o emulsions resemble bulk oils in their response to the polarity of compounds. In multiphase systems such as emulsions, there are interactions between iron, emulsifiers and antioxidants.

	away from the interface. The polar paradox was insufficient to explain the antioxidant effects in the multiphase systems.	
Sun *et al.*, 2011	Polar antioxidants with higher affinity were known to concentrate on oil/air or oil/water interfaces of the reversed micelle. The antioxidant polar paradox does not always prevail. The influencing factors of antioxidant activity in reversed micelle were not solely based on antioxidant polarity.	Polar paradox is affected by other factors contributing to non-linearity in the effect of antioxidants in lipid oxidation.
Sun-Waterhouse *et al.*, 2011	Caffeic acid and p-coumaric acid are hydrophilic. They tend to partition into the water phase, locate outside of the oil droplets and chelate metal ions which exist in the oils. Both antioxidants stabilized oil against autoxidation but caused TAG hydrolysis.	Antioxidants may cause other adverse effects, e.g. hydrolysis of TAG.
Chen *et al.*, 2012a	Soybean oil is found in seeds inside micro-sized oil bodies, which consist of a central neutral lipid core (94-98% w/w) and is surrounded by phospholipids monolayer (0.5-2% w/w) and a coat of strong amphiphilic oleosin (0.5-3.5% w/w). These soybean oil bodies had a better physicochemical stability than emulsified soybean oil. Heat treatment (55°C) did not affect LOOH and hexanal in oil body suspensions (2% wt at pH 3).	Natural organization protects unsaturated fatty acids. Water exists as nano-scale droplets in w/o emulsions.
Rukmini *et al.*, 2012	W/o microemulsion exist in bulk oil with nano-scale droplets of water inside. Stabilization of water-in-virgin coconut oil were prepared with food grade nonionic surfactants (Span 80, Span 20 and Tween 20). Cosurfactants may not be suitable for foods because of the toxicity and irritation induced by short- and medium-chain alcohols. Nonionic surfacts stabilized w/o emulsion without the use of cosurfactants, but there was phase separation at 70°C or higher.	Nonionic surfactants offer an alternative solution as it stabilizes w/o emulsions and do not contribute to oxidation.

The first research which investigated the effects of molecular properties other than BDE was proposed (in the pioneering papers) by William Porter (Porter, 1980; Porter *et al.*, 1989; and Porter, 1993). He proposed the *polar paradox* of antioxidants. That is, polar or hydrophilic antioxidants (such as Trolox C, ascorbic acid, propyl gallate and TBHQ) are more efficient in protecting bulk lipids with a low surface/volume ratio, whereas the nonpolar or lipophilic antioxidants (e.g. α-tocopherol, ascorbyl palmitate, BHA and BHT) are more efficient in protecting oil-in-water emulsions (o/w) with a high surface to volume ratio against oxidation. This polar paradox was further elucidated by the interfacial phenomena theory proposed by Frankel and coworkers (Frankel *et al.*, 1994, 1996a,b). The theory gave a reasoning that hydrophilic antioxidants have more affinities to and thus are more partitioned at the air-oil interfaces and protect bulk oils, whereas the lipophilic ones had more affinities at the water-oil interfaces in o/w. This interfacial phenomenon was first investigated in o/w emulsions due to the greater availability of the analysis methods used to study such emulsions, however, this was not the case with the bulk oils (Chaiyasit *et al.*, 2007). The interfacial phenomena theory was later rectified by later studies and will be discussed later in other parts of this Chapter. Since then, many studies have been performed utilizing antioxidants with different polarities which have resulted in different effectiveness in bulk lipids, and thus the polar paradox theory was confirmed. Some of these pairs of antioxidants, for instances carnosic acid vs. methyl carnosate (Huang *et al.*, 1996a and Schwarz *et al.*, 2000), and quercetin vs. rutin (Wanasundara and Shahidi, 1998 and Becker *et al.*, 2007) are presented in **Table 2.2** and **Figure 2.1**. The following are some examples, elucidating the different polarities of antioxidants and their effects in the antioxidants effectiveness in bulk oils: esterification of sinapic acid reduces its radical-scavenging activity (Thiyam *et al.*,

2006), conjugation and steric hindrance affect molecular planarity and thus antioxidant effectiveness of caffeic acid, dihydrocaffeic acid, and rosmarinic acid (Nenadis *et al.*, 2003), the existence of a double bond in the acyl substituent decreases the polarity and antioxidant capacity of propyl caffeate compared to hydrocaffeate, and of ferulate compared to isoferulate (Silva *et al.*, 2001), alkylation of ferulic acid improves its solubility in linoleic acid but reduces its antioxidant activity (Fang *et al.*, 2006). Catechin with *trans* configuration is a better antioxidant compared to epicatechin with *cis* configuration (Huang and Frankel, 1997), lipophilic methyl carnosate is a less effective antioxidant than its polar counterpart carnosic acid (Huang *et al.*, 1996a and Schwarz *et al.*, 2000), and the addition of an alkyl chain in hydroxytyrosol fatty acid esters causes it to be more lipophilic and thus lowers the compound effectiveness (Trujillo *et al.*, 2006 and Medina *et al.*, 2009).

Even though the polar paradox has been evidenced in many studies, some of the effects of antioxidant polarity on its effectiveness in bulk oils have been variable, which indicates that the polar paradox is not always accurate. It was shown in a study with epigallocatechin-gallate (EGCG) and its esters (Shahidi and Zhong, 2011) and glycosylation of quercetin (such as quercetin-3-O-glucoside and rutin) (Wanasundara and Shahidi, 1998; Becker *et al.*, 2007; and Huber *et al.*, 2009), that the lipophilic antioxidants are more effective in bulk oils when added at lower levels compared to their hydrophilic ones, because the effects of the solubility of the antioxidant is more important than the interfacial phenomenon at these lower levels. This suggested that the polar paradox might prevail only when an antioxidant is applied at a high level reaching its critical concentration, where the interfacial phenomena dominates over solubility effects (Shahidi and Zhong, 2011). Further discussions are available in the next parts of this chapter.

Table 2.2. Effects of pairs of antioxidants with similar structure and different hydrophilicity in bulk oils

References	Antioxidants	Substrates and conditions	Results
Frankel *et al.*, 1994	Ascorbic acid vs. ascorbyl palmitate	Stripped corn oil, added antioxidant (232 and 1161 μM), 60°C	Ascorbic acid was a more potent antioxidant than ascorbyl palmitate based on LOOH and hexanal formation.
Carelli *et al.*, 2005		SFO; each additive is at 200, 400, 600 and 800 ppm; 30 and 68 °C (Oven) and 130 °C (Rancimat)	Ascorbic acid was a more effective antioxidant than ascorbyl palmitate, according to Rancimat, and peroxide value (PV) (30 °C), para-anisidine value (p-AV), total content and distribution of polar compounds and residual α-tocopherol.
Sorensen *et al.*, 2011		w/o emulsion (98% of 1:1 fish oil:rapeseed oil, stripped), 1% polyglycerol polyricinoleate emulsifier; antioxidants added at 100 mM; 37°C	Ascorbic acid was a more effective antioxidant than ascorbyl palmitate on the basis of LOOH and propanal formation. Ascorbyl palmitate exhibited pro-oxidative effects toward the end of the storage period.
Frankel *et al.*, 1994	Trolox vs. α-tocopherol	Stripped corn oil, antioxidants added at 232 and 1161 μM, 60°C	Trolox was a better antioxidant than α-tocopherol on the basis of LOOH and hexanal formation. At a high concentration, α-tocopherol had a prooxidant effect.
Schwarz *et al.*, 2000		Stripped corn oil; w/o emulsion; ceteareth-15 and glyceryl stearate, polyglyceryl glucose methyl distearate, polysiloxan polyalcohol polyether copolymer and polyglyceryl-3 oleate as emulsifers each at 20% level; antioxidants added at 100 mM (based on the oil phase), 37 and 60°C.	Trolox had higher activity than α-tocopherol based on LOOH and hexanal formation in both bulk oil and w/o emulsion with and without emulsifers. With a few exceptions, α-tocopherol showed better activity for w/o polysiloxan polyalcohol polyether copolymer emulsion based on LOOH and w/o polyglyceryl-3 oleate emulsion based on hexanal formation at 37°C. α-tocopherol showed a prooxidative effect in bulk oil with polyglyceryl-3 oleate at 60 °C.
Huang *et al.*, 1996a		Linoleic acid, methyl linoleate, corn oil TAG, α-tocopherol are at 65 and 130 ppm and Trolox are at 38 and 76 ppm, 37°C or 60°C in a shaking water bath.	Trolox was a better antioxidant than α-tocopherol, in terms of LOOH and hexanal formation. With a few exceptions, α-tocopherol showed a better activity than Trolox (38 ppm), at 37°C, in bulk methyl linoleate and corn oil TAG based on hexanal formation; and at 60°C in bulk corn oil TAG, Trolox caused a pro-oxidative effect by hexanal results.

Chen *et al.*, 2011b		Stripped SBO; 1,2-dioleoyl-sn-glycero-3 phosphocholine, 1,2-dibutyryl-sn-glycero-3-phosphocholine each at 1000 μM, antioxidants at 10 and 100 μM; 55°C.	Trolox was a more effective antioxidant than α-tocopherol with and without the addition of phospholipids (except 1,2-dibutyryl-sn-glycero-3-phosphocholine). 1,2-dioleoyl-sn-glycero-3-phosphocholine improved, while 1,2-dibutyryl-sn-glycero-3-phosphocholine decreased the α-tocopherol and Trolox activity.
Thiyam *et al.*, 2006	Sinapic acid and sinapine	Purified rapeseed oil, sinapic acid and sinapine at 50 and 500 μmol/kg oil, 40°C.	Sinapic acid was better in reducing LOOH and propanal compared to its derivatives sinapine.
Chen and Ho, 1997	Caffeic acid vs. caffeic acid phenethyl ester	Lard and corn oil each at 2 mM, Rancimat (110°C and 20 mL/min).	In lard, caffeic acid had better activity in extending the IP than caffeic acid phenethyl ester did. In corn oil, the activities of both antioxidants were the same.
Nenadis *et al.*, 2003	Caffeic acid vs. dihydrocaffeic acid	Triolein, each additive is at 10 ppm, 45°C in the dark	Based on the PV, the activity dihydrocaffeic acid was higher than caffeic acid and control. The presence of the conjugated double bond in the side chain of caffeic acid, makes its less polar and also decreases its hydrogen-donating properties, compared to dihydrocaffeic acid.
Silva *et al.*, 2001	Propyl caffeate and hydrocaffeate	Refined SFO, propyl hydrocaffeate and propyl caffeate each at 160 and 200 ppm, Rancimat 110°C and 20 L/h	The antioxidant effectiveness of propyl hydrocaffeate was higher than that of propyl caffeate.
Leonardis *et al.*, 2008	Caffeic acid vs. chlorogenic acid (ester of caffeic acid and quinic acid)	Cod liver oil, antioxidants added at 0.005 - 0.05 % by weight, Rancimat at 80 and 100°C and 20 L/h	Caffeic acid was a better antioxidant compared to chlorogenic acid. The latter exhibited weak antioxidative effects.
Laguerre *et al.*, 2011	Chlorogenic acid and its esters	Stripped corn oil, each additive is at 200 μmol/kg, 55°C in the dark	Hydrophobicity of chlorogenic acid and its butyl, dodecyl and hexadecyl esters did not correlate well with their antioxidant capacity in bulk oil. With and without dioleoylphosphatidylcholine, conjugated dienes test showed a longer IP as the akyl chain increased.
Chen and Ho, 1997	Ferulic acid vs. ferulic acid phenetyl ester	Lard and corn oil, 2 mM, Rancimat (110°C and 20 mL/min)	In lard, ferulic acid and ferulic acid phenetyl ester had the same activity in extending the IP. In corn oil, both compounds had no significant effects in improving the oxidative stability.

Fang *et al.*, 2006	Ferulic acid vs. alkyl ferulates: 1-pentyl, 1-hexyl, 1-heptyl ferulates	Linolec acid; each additive is at 1.0 x 10^{-4}, 3.0 x 10^{-4}, 1.0 x 10^{-3}, 3.0 x 10^{-3} molar ratios of the additive to linoleic acid; 37, 50, 65, 80°C in the dark	Alkyl ferulate (1-pentyl, 1-hexyl and 1-heptyl ferulates) slightly increased the antioxidant activity compared to ferulic acid but their activities were not significantly different.
Silva *et al.*, 2001	Propyl ferulate and isoferulate	Refined SFO, propyl isoferulate and propyl ferulate each at 160 and 200 ppm, Rancimat 110°C and 20 L/h	Propyl isoferulate had a better antioxidant activity than propyl ferulate.
Huber *et al.*, 2009	Quercetin and quercetin-3-O-glucoside	Fish oil without antioxidant; each additive is at 100, 500, 1000 and 5000 μM; 70°C	Quercetin-3-O-glucoside had a higher antioxidant activity than quercetin at 100 and 500 μM. At 1000 μM, their activities were equal.
Becker *et al.*, 2007	Quercetin vs. rutin	Purified high-oleic SFO; each additive is at 0.25, 0.5, 1.0 and 2.0 mmol /Kg oil; Rancimat 100°C and 20 L/h	The antioxidant activity of quercetin was higher than rutin.
Wanasundara and Shahidi, 1998		Refined-bleached and deodorized seal blubber oil and menhaden oil, each additive is at 200 ppm, 65°C in Schaal oven	The antioxidant activity of quercetin was higher than rutin in all substrates, as monitored by weight gain, PV and thiobarbituric acid reactive substances (TBARS).
Huang and Frankel, 1997	Catechins	Corn oil TAG, each additive is at 140 μM, 50°C	According to LOOH formation: gallic acid had more antioxidant activity than epicatechin gallate and epigallocatechin gallate (EGCG) had more antioxidant activity than epigallocatechin.
Shahidi and Zhong, 2011	EGCG and its esters	Stripped corn oil; 1 ml / 3 g, Rancimat (100°C and 20 L/h)	At lower concentrations, the antioxidant activity of EGCG was lower than its lipophilic ester derivative (stearate); but the effects were reversed at higher concentrations.
Huang *et al.*, 1996b	Carnosic acid and methyl carnosate	Corn oil TAG, each additive is at 150 and 300 μM, 60°C	The antioxidant activity of methyl carnosate was higher than carnosic acid on basis of LOOH and hexanal formation.
Schwarz *et al.*, 2000		Tocopherol-stripped corn oil; water in oil emulsion; ceteareth-15, glyceryl stearate, polyglyceryl glucose methyl distearate, polysiloxan polyalcohol polyether copolymer, and polyglyceryl-3 oleate emulsifers at 20% level; antioxidants at 100 mM, 37 and 60°C	Methyl carnosate had higher antioxidant activity compared to carnosic acid in both bulk oil and w/o emulsions according to LOOH and hexanal formation, which does not comform to the polar paradox.

Frankel *et al.*, 1996a	Carnosic acid and carnosol	Stripped corn oil, each additive is at 50 ppm, 60°C in a shaker oven	Carnosic acid had a higher antioxidant activitiy than carnosol in bulk oil on the basis of LOOH and hexanal formation.
Frankel *et al.*, 1996b		Corn oil, SBO, peanut oil, and fish oil. Each additive is at 30 and 50 ppm, 60°C	Carnosic acid had a higher antioxidant activitiy than carnosol in bulk corn oil, SBO, peanut oil, and fish oil on the basis of conjugated dienes and hexanal formation.
Hopia *et al.*, 1996		Methyl linoleate, linoleic acid, corn oil TAG, additives at 150 and 300 μM, 37 and 60°C	Carnosic acid was a better antioxidant than carnosol in methyl linoleate and corn oil but not in bulk linoleic acid where carnosol had better activity than carnosic acid. The substrate seems to affect the performance of antioxidants.
Trujillo *et al.*, 2006	Hydroxytyrosol and its fatty acid esters	Glyceridic matrix, each additive is at 1 and 5 mM, Rancimat 90°C	Hydroxytyrosol had higher antioxidant activity than hydroxytyrosyl acetate, palmitate, oleate and linoleate.
Medina *et al.*, 2009		Fish oil, each additive is at 10, 25, 50, 100, 150 and 200 ppm, 40°C	Hydroxytyrosol had better antioxidant activity than its esters with increasing size of alkyl chain (i.e. hydroxytyrosol acetate, butyrate, octanoate, laurate and octyl gallate).
Medina *et al.*, 2010	Resveratrol vs. acylated and glucosylated resveratrol	Cod-liver oil, each additive is at 100 ppm, 40°C	Resveratrol fatty acid esters with increasing size of alkyl chain (i.e. 3-stearoylresveratrol, 3-stearoylresveratrol and 4'-stearoylresveratrol) and glucosylation (i.e. resveratrol-3-β-D-glucopyranoside, resveratrol-3,5-di-β-D-glucopyranoside and resveratrol-3,4'-di-β-D-glucopyranoside) had reduced antioxidant effectiveness compared to original phenol.

2.4 The role of microenvironments (micelles) on oxidation

Interfacial phenomenon of Frankel *et al.*, (Frankel *et al.*, 1994 and 1996a and b) was explained further by Koga and Terao (1995), who suggested that phospholipids increased the antioxidant activity of α-tocopherol by assisting the tocopherol to the microemulsions or the oxidation site. Koga and Terao (1995) recognized that the lipid oxidation is dominant at the interfaces formed between traces of water and oils (where radicals are formed and trapped) rather than at the interfaces between air and oils as suggested by Frankel and coworkers (Frankel *et al.*, 1994 and 1996a and b).

Phospholipids are amphiphiles and can improve the partition of α-tocopherol in the water phase of the microemulsions or reversed micelles. The phenolic head of α-tocopherol and the polar head of phospholipids are located near the polar region of the reversed micelles where radicals are formed and trapped, while the nonpolar acyl chains are located in the oil phase (Koga and Terao, 1995). The roles of micelles in oxidation has also been studied by Ulla Brimberg (1993), who suggested that the transition from induction to propagation period is governed by the critical micelle concentration (CMC) that is formed by hydroperoxides and influenced by amphiphiles such as antioxidants, synergists and prooxidants (Brimberg, 1993a and b). Brimberg (1993a and b) also proposed a set of empirical equations to describe the oxidation of different oil/additive combinations satisfactorily. Brimberg and Kamal-Eldin (2003b) proposed that lipid oxidation in bulk oils follows a pseudo-first order in the beginning when hydroperoxides are gradually formed until their CMC is reached. Then it starts to aggregate to form reversed micelles and the reaction rate becomes a second order and oxidation enters the propagation stage (**Figure 2.3**). As a result, it can be seen that the main effects of anti- and prooxidants depend on their roles to modulate the CMC of lipid hydroperoxides.

Studies of lipid oxidation in o/w emulsions found some interesting results. They showed properties of emulsion droplets and interface properties (e.g. droplet size, interfacial area, charge, thickness and permeability) affect lipid oxidations in the emulsions (Mancuso *et al.*, 1999; Chaiyasit *et al.*, 2007; and Yi *et al.*, 2011). For example, cationic charge at the interfaces causes transition metals to be repelled and thus lowers the rate of oxidation in o/w (Chaiyasit *et al.*, 2007). This was later investigated in depth and verified with supporting results by the research group of

Decker and co-workers (McClements and Decker, 2000; Chaiyasit *et al.*, 2007; and Yi *et al.*, 2011).

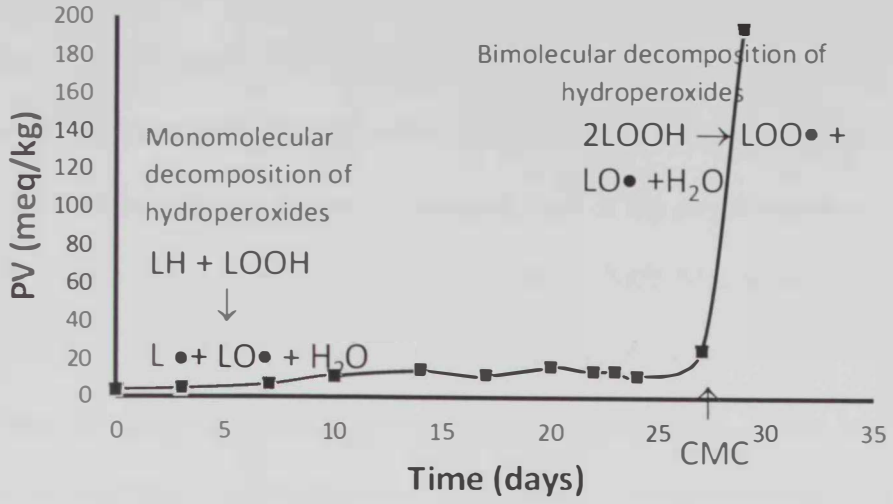


Figure 2.3. Changes of peroxide value (PV) during the autoxidation of flaxseed oil at 40°C. The first period (lag phase or induction period) is dominated by reactions between unsaturated fatty acids (LH) and low concentrations of hydroperoxides (LOOH). This reaction is first order with respect to LH and LOOH but because of the relatively very high concentration of LH and the very low concentration of LOOH, it is often described as zero order. When the concentration of LOOH reaches its critical micelle concentration (CMC), formation of micelles become significant and the reaction enters the bimolecular phase with respect to hydroperoxides. Attainment of CMC marks the end of the induction period (IP).

In nonpolar mediums such as bulk oils, when surface active compounds (e.g. lecithin and other polar minor components) present with small amount of water, association colloids (such as reversed micelles) are formed and play a role as oxidation reaction sites (Chaiyasit *et al.*, 2007 and 2008). The kinds of association colloids and the effects of amphiphilic compounds depend on their physicochemical properties, such as hydrocarbon chain length (which influences their hydrophilic lipophilic balance (HLB) (Koga and Terao, 1995) and quantities (King *et al.*, 1992b and Subagio and Morita, 2001). These surface active compounds and micelle entities can change the physical location of other compounds and hence affect oxidation rates in bulk oils

(Chaiyasit *et al.*, 2008 and Chen *et al.*, 2011b). The evidence that these microenvironments are oxidation sites was explained by the facts that oxygen (the primary catalyst of lipid oxidation) is 3-10 times more soluble in oil than in water and air, and is more nonpolar than oil (the dielectric constants of air and oil are 1.0 and 3.0, respectively). Thus, hydrophilic antioxidants are more likely to be located at the oil-water interface of the microenvironments, than at the air-oil interface (Hopia *et al.*, 1996; Huang *et al.*, 1996a and b; Chaiyasit *et al.*, 2007; Shahidi and Zhong, 2011; and Sorensen *et al.*, 2011) and arrange to form microemulsions and create a barrier to protect the micelles (Chaiyasit *et al.*, 2007 and Lucas *et al.*, 2010). In a similar way, higher quantities of emulsifiers (such as lecithin), form micelles and bring antioxidants closer to the polar phase or interface (Pena and Miller, 2006 and Oehlke *et al.*, 2010). In the case of lipophilic antioxidants, they disperse or stay in the continuous oil phase and do not concentrate at the catalytic sites of the micelles. As a result, these antioxidants are less effective in protecting bulk lipids against oxidation. Lipophilic antioxidants protect more o/w emulsions because these antioxidants orient more at the water-oil interface of the emulsions (Frankel *et al.*, 1994; Chaiyasit *et al.*, 2005 and 2008; and Sorensen *et al.*, 2011).

Different effects of additives on the oxidation of bulk oils are presented in **Table 2.3**. Some explanations include the fact that the more hydrophilic Trolox exhibited more protection than α-tocopherol in bulk oils because the former is more concentrated in the water phase of the mixed micelles (Aubourg, 2001 and Chen *et al.*, 2010). In the other cases, trace metal ions (Mei *et al.*, 1998 a and b and Mancuso *et al.*, 1999), free fatty acids and monoacylglycerols act as prooxidants by the micellar effect or by migrating at the oil-water interface, thus accelerating the decomposition of hydroperoxides (Frankel *et al.*, 1994; Chaiyasit *et al.*, 2005 and 2008; and Sorensen *et*

al., 2011). In the case of phospholipids, they act as synergists to primary antioxidants by moving them closer to the interface (Chaiyasit *et al.*, 2007 and 2008). Primary antioxidants and phospholipids trap and inhibit the diffusion of radicals into the bulk oils by volume cage effects (Kortenska *et al.*, 2002), wherein the polar heads of the two are positioned at the oil/water interface of the microenvironment or cage (Koga and Terao, 1995) and hydrogen atoms are given to radicals, hence retarding the oxidation. The combinations of antioxidants and surfactants can result in synergistic or antagonistic effects (**Figure 2.2**), depending on their hydrocarbon chain length, hydrophilic lipophilic balance and levels in the oils (Pena and Miller, 2006 and Oehlke *et al.*, 2010).

As a summary, microenvironments (i.e. microemulsions) act as nanoreactors for the hydroperoxides autocatalysis, thus creating the oxidation site. The oxidation reactions are predominant at the oil/water interfaces, and any changes in the regions can increase or decrease the reaction rate. For instance, antioxidants that are partitioned near the polar region will scavenge radicals in the regions where oxidation is prevalent (Kiokias and Gordon, 2003; Decker *et al.*, 2005; Kasaikina *et al.*, 2008; Koprivnjak *et al.*, 2008 and Sun-Waterhouse *et al.*, 2011). Moreover, the nature of these microenvironments are influenced by and also can modify the physical location of prooxidants (such as oxidation products), antioxidants and synergists. Therefore, these microenvironments affect the rate of lipid oxidation (Chaiyasit *et al.*, 2007).

Table 2.3. The influence of different kinds of additives on the protection of bulk lipids

References	Substrates & Oxidation Conditions	Additive(s)	Results	Conclusions
Fatty alcohols				
Yanishlieva and Kortenska, 1989	SFO, OO, lard, tristearin, olive oil methyl esters, 70-135°C	1-tetradecanol, 1-hexadecanol, 1-eicosanol (5-80 mmol / kg)	The pro-oxidative effects of fatty alcohols depend on the type, concentration, valency of the alcohols and LOOH, and the degree of unsaturation of the lipid media. The pro-oxidative effect was less in TAG than in fatty acid methyl esters.	Fatty alcohols antagonized the antioxidant effect of phenolic inhibitors.
Kortenska *et al.*, 1991	SFO, methyl ester, 50°C	p-methoxyphenol (0.1 M), 1-octadecanol (0.1 M), 1-palmitoylglycerol (0.1 M)	1-octadecanol and 1-palmitoylglycerol acted as a prooxidant, by decreasing the rate constant of chain termination, in the presence of an inhibitor (p-methoxyphenol).	Fatty alcohols inhibited inhibitor by formation of H-bonds and complex formation. 1-palmitoylglycerol had a stonger effect because of its two hydroxyl groups.
Yanishlieva and Kortenska, 1993	TAG of SFO and TAG of OO, 23 and 110°C	Hydroquinone (1×10^{-4} mol/l), 1-tetradecanol, 1-octadecanol ($(0.5\text{-}9.0) \times 10^{-2}$ mol/l)	Fatty alcohols accelerated the oxidation of lipids (in the presence of hydroquinone). Increasing unsaturation of substrate caused a lesser prooxidative effect on the alcohols.	Shorter chain alcohols caused stronger complex formation (H-bond) with hydroquinone. Longer chain alcohols had higher prooxidative activity in the propagation, branching and termination reactions.
Kortenska and Yanishlieva 1995	TAG of SFO, 80°C	Hydroquinone, BHT, α-tocopherol (each at 0.1 mM); 1-tetradecanol, 1-octadecanol (5, 20, 40, 60 mM)	Fatty alcohols were prooxidants. A linear dependence of oxidation of oil was seen, with hydroquinone, in the presence of 1-tetradecanol, α-tocopherol + 1-tetradecanol + 1-octadecanol. There were no interactions	There was no interaction between 1-octadecanol and BHT, because 1-octadecanol participates in the process only by accelerating the decomposition of LOOH.

			between BHT and 1-octadecanol in inhibiting oxidation.	
Kortenska *et al.*, 2001 and 2002	TAG of SFO, 80°C	2,6-di-tert-butyl-4-methylphenol (0.1 mM), 1-tetra-decanol (40 mM), 1-octadecanol (40 mM), and 1-mono-palmitoyl-glycerol (40 mM)	Fatty alcohols decreased the IP, as measured by LOOH. 1-monopalmitoylglycerol caused a further reduction of IP. BHT + fatty alcohols also exhibited prooxidative effects, with no improvement on the IP.	Polar compounds such as fatty alcohols and oxidation products associate in non-polar medium. LOOH decomposed faster inside the polar interior of the micro emulsion. Fatty alcohols alone and combined with BHT were prooxidants.
Kortenska *et al.*, 2002	SFO and lard, 100°C	α-tocopherol (1.3 mM), 1-octadecanol (5, 40, 80 mM)	The higher the 1-octadecanol level, the more the reduction of IP and increase of oxidation rate in both medium. The relative increase of oxidation rate in SFO was more than in lard.	Fatty alcohols acted as prooxidant and inhibited α-tocopherol activity.
Free Fatty Acids (FFA)				
Miyashita and Takagi, 1986	Oleic acid, methyl oleate, linoleic acid, methyl linoleate, linolenic acid, methyl linolenate; 50°C	No additives	Methyl esters are more stable than their corresponding FFA (longer IP and have lower PV).	FFAs are more susceptible to oxidation than corresponding esterified fatty acids. The catalytic effect of the carboxyl groups of FFA on the formation of free radicals and decomposition of LOOH was thought to be the reason. According to the current theory, a bulk of FFAs is different from a bulk of TAGs in the molecular assembly of the substrate itself and of any added additive. FFAs are surface active and contribute to
	Methyl linoleate and SBO; 50°C in the dark	Stearic acid (0, 0.5, 1, 3 and 5%)	The addition of stearic acid to methyl linoleate or SBO did not affect the IP but increased the oxidation rate during this IP.	
	Methyl linoleate hydroperoxides; 50°C in the dark	Stearic acid (0, 0.2, 0.5 and 1%)	Hydroperoxides (PV and conjugated diene content), decomposed faster in the presence of stearic acid.	
Mistry and Min., 1987b	SBO, forced air oven 55°C	Stearic, oleic, linoleic, linolenic, or octadecane (0, 0.5 and 1%)	FFA, but not octadecane, showed prooxidant activity in SBO (PV, volatile compounds, and	

			oxygen in the headspace).	micelle formation when added to TAGs.
Hamam and Shahidi, 2004	Doxosahexaenoic acid single cell oil; Schaal oven 60°C	Capric acid (not added but due to acidolysis)	Acidolysis of the single cell oil, with capric acid, decreased the oxidative stability of the oil (Conjugated dienes and TBARS) compared to unmodified doxosahexaenoic acid single cell oil.	
Frega *et al.*, 1999	Virgin OO; Rancimat 110°C, 20 L/h	Oleic acid or methyl oleate (0-3%)	Methyl oleate but not oleic acid (% acidity) decreased the IP of virgin OO.	The prooxidant effect of FFA is dependent on the matrix. For example, oleic acid had a prooxidant activity in membrane-filtered and bleached OO but not in cloudy oils; and methyl oleate but not oleic acid had a prooxidant effect on virgin OO.
	OO (cloudy untreated, cloudy paper-filtered, cloudy membrane-filtered, and cloudy bleached with clay); Rancimat 110°C, 20 L/h	Oleic acid (0-3%)	Oleic acid (% acidity) increased the IP of cloudy untreated oil, but did not affect the IP of cloudy paper-filtered oil, and decreased the IP of cloudy membrane-filtered and cloudy bleached oils.	
Chaiyasit *et al.*, 2007	Methyl linolenate in model oil system containing sodium bis(2-ethylhexyl) sulfosuccinate, water-hexadecane; ferrous sulfate, 24°C in the dark	Oleic acid (0, 25, 50 and 100 mmol/kg lipid)	Oleic acid reduced the reversed micelle size and accelerated lipid oxidation (LOOH and TBARS) compared to a control and added phosphatidylcholine.	The prooxidant effect of FFA seems to be related to their action as surfactants (increasing the number of small micelles).
Monoacylglycerols (MAG) and diacylglycerols (DAG)				
Mistry and Min, 1987a	Refined, bleached and deodorized SBO, forced-air oven 55°C	1-monolinolein (0.01%)	MAG (1-monolinolein) had prooxidant activity in SBO (PV and volatile compounds).	MAG and DAG showed prooxidant effects in pure TAG depending on their polarity,

Mistry and Min, 1988	SBO, forced-air oven 55°C	Monostearin, monolinolein, distearin and dilinolein (0, 0.25 and 0.5%)	Monostearin, monolinolein, distearin and dilinolein acted as prooxidants in SBO (decrease of headspace oxygen) in a concentration-dependant manner.	concentration and temperature.
Caponio *et al.*, 2011	Purified SBO, oven 60°C; measured at 4, 6, 9, 14 and 18 days	Unnamed MAG (0, 0.5, 1, 2 and 3%)	The effect of MAG in the oxidation of purified SBO is concentration-dependent. At low amounts (0.5 & 1%), MAG increased the oxidation rate while at higher concentrations the IP was reduced.	
Aubourg, 2001	Cod liver oil containing citric acid; 15, 30, 50°C	Unnamed MAG (0.1, 0.5, 2, 5 %)	MAG (2 & 5%) caused an inhibitory effect on the protective effect of citric acid at 50°C, but not at 15 and 30°C.	MAG and DAG enhanced the oxidation of cod liver oil containing citric acid, which functions as a metal chelator. A longer chain length increased the prooxidant effect possibly because of increased surfactant activity.
	Cod liver oil containing citric acid; 50°C	Monolauroyl-glycerol, monomiristoyl-glycerol, monopalmitoyl-glycerol and monostearoyl-glycerol (3.78 mM)	The prooxidant effect of MAG increased as the chain length of MAG increased.	
	Cod liver oil containing citric acid; 30, 50°C	Unnamed diacylglycerols (0.1, 0.5, 2, 5 %)	DAG showed an inhibitory effect on the protective effect of citric acid at 50°C but not at 30°C. There was no difference in effect between different DAG concentrations.	
Wang *et al.*, 2005	Purified and natural corn oil; 28°C	1-monolinoleoyl-rac-glycerols (0, 0.1, 0.25, and 0.5%)	MAG decreased the IP for purified but not for unpurified oil.	MAG may contribute pro-oxidant effect(s) in the oxidation of bulk oils.
	Natural and randomized corn oil, Oxidative Stability Index (OSI), 28°C	1-monolinoleoyl-rac-glycerols and 1,3-dilinoleoyl-rac-glycerol (conc. unknown)	MAG showed a higher prooxidant activity than DAG (reduced OSI IP).	
	Natural and randomized	1,3-dilinoleoyl-rac-glycerol (5%)	There was an increase in the oxidation rate of purified oil with 5%	

	corn oil; 28°C		DAG but the increases were not as great as that of randomized oil.	
Phospholipids (PL)				
King *et al.*, 1992b	Salmon oil, Fischer forced-draft oven, 180°C	Phosphatidylcholine (0.01, 0.1 and 1%)	Phosphatidylcholine improved the oxidative stability of the oil in a concentration-dependant manner (as measured by TBARS and polyene index).	The amine group of phosphatidylcholine and phosphatidylethanolamine and reducing sugar of phosphatidylinositol can facilitate hydrogen or electron donation by α-tocopherol at 180°C. Nitrogen-containing phospholipids perform better in improving the oxidative stability of oil than those that do not contain nitrogen.
	Salmon oil, Fischer forced-draft oven, 180°C	Phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine, sphingomyelin (1% each)	Nitrogen-containing phospholipids (i.e. phosphatidylethanolamine, phosphatidyl-choline, lysophosphatidylcholine & sphingomyelin had higher antioxidant acitivity than phosphatidylglycerol & phosphatidylinositol (yielded the least activity). The slope of oxidation rate showed that sphingomyelin was the most effective and phosphatidylinositol was the least effective.	
	Methyl linoleate, methyl laureate, 50°C in the dark, continuous shaking at 120 rpm	Dipalmitoylphosphatidylcholine (DPPC, 100 nM), dipalmitoyl-phosphatidylethanol-amine (DPPE, 100 nM), α-tocopherol (10 nM)	Without α-tocopherol, Dipalmitoylphosphatidylcholine gave a slower oxidation rate than dipalmitoylphosphatidylethanolamine. With α-tocopherol, IP was prolonged and methyl linoleate-OOH accumulated after α-tocopherol was consumed. DPPC and DPPE showed an insignificant effect in oxidation rate, in the presence or absence of α-tocopherol.	DPPC and DPPE act synergistically with α-tocopherol, but the effect is insignificant.
Koga and Terao, 1995	Methyl linoleate; methyl laureate;	Dipalmitoylphosphatidylcholine, dibutyrylphosphatidylcholine,	With the use of 2,2'-azobis (2-amidinopropyl) dihydrochloride,	Phospholipids accelerated the consumption of α-tocopherol when

	2,2'-azobis(2-amidinopropyl) dihydrochloride (AAPH) (water soluble radical initiator); 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN) (lipid soluble radical initiator), 50°C, shaking at 120 rpm	dicaprylphosphatidylcholine, dimyristoylphosphatidylcholine (each 100 nM), α-tocopherol (10 nM)	phospholipids caused a rapid consumption of α-tocopherol. With 2,2'-azobis (2,4-dimethylvaleronitrile), phospholipids did not affect the consumption of vit E. The IP increased with an increase in hydrocarbon chain length of acyl moieties of phospholipids.	radicals were generated from water-soluble radical generators with a trace of water.
Khan and Shahidi, 2000	Borage oil TAG, dark, in a Schaal oven at 60°C	Phosphatidylcholine (500 ppm), phosphatidylethanolamine (500 ppm), α-tocopherol (500 ppm), δ-tocopherol (500 ppm)	Phosphatidylcholine lengthened the oxidation time more than phosphatidylethanolamine (based on conjugated dienes). Combinations of phosphatidylcholine + α-tocopherol., phosphatidylcholine + δ-tocopherol, phosphatidylethanolamine + α-tocopherol, phosphatidylethanolamine + δ-tocopherol (each was 500 ppm) lengthened the oxidation time more than individually added phosphatidylcholine, phosphatidylethanolamine, α-tocopherol and δ-tocopherol. Combination of α-tocopherol with each phospholipid was more effective than a combination of δ-tocopherol. The most effective combination	Phosphatidylcholine is more effective than phosphatidylethanolamine alone and in combination with α-tocopherol in borage oil TAGs. Phosphatidylethanolamine was more effective than phosphatidylcholine in evening primose TAGs. Phospholipids increased the accessibility of the tocopherols to the aqueous environment (the micellar phase).

			was that of phosphatidylcholine and α-tocopherol (on basis of TBARS).	
	Evening primrose oil TAG, dark, in a Schaal oven at 60°C	Phosphatidylcholine (500 ppm), phosphatidylethanolamine (500 ppm), α-tocopherol (500 ppm), δ-tocopherol (500 ppm)	Phosphatidylethanolamine lengthened the IP more than phosphatidylcholine (based on conjugated dienes). Combinations of phosphatidylcholine + α-tocopherol, phosphatidylcholine + δ-tocopherol, phosphatidylethanol-amine + α-tocopherol, phosphatidylethanol-amine + δ-tocopherol (500 ppm phospholipids and 500 ppm tocopherol) with the combinations with phosphatidylethanolamine being more effective than those with phosphatidylcholine (on basis on TBARS).	
Hidalgo *et al.*, 2005	Refined SBO, heated in the dark under air at 60°C	Phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol (each 200 ppm)	Phospholipids lengthened the IP (phosphatidylcholine > phosphatidylethanol-amine > phosphatidylinositol). The protection was better with oxidized phosphatidylethanol-amine, phosphatidylcholine, & phosphatidylinositol. Phosphatidylethanol-amine showed max. antioxidative activity when the pyrrole content was between 800-1400 nmol of pyrrole/mmol of phosphatidylethanol-amine.	Phosphatidylcho-line,phosphatidyl-ethanolamine, & phosphatidylino-sitol improved the oxidative stability of the oil. The antioxidant was improved in combination with phosphatidyl-ethanolamine or phosphatidylcho-line (synergism). No synergism was observed for phosphatidylcho-line plus phosphatidyl-ethanolamine.
	Refined OO (ROO),	Phosphatidylethanol-amine, phosphatidyl-choline, lysine, BHT (each added at 4	Phosphatidylethanol-amine, phosphatidyl-choline, lysine, and BHT increased the	

	Rancimat 110°C	levels: 100, 200, 300 and 400 ppm)	oxidative stability of the oil at 200 ppm or more. The IP of oil with 400 ppm phosphatidylethanol-amine had a similar stability as that of oil with 200-400 ppm BHT.	
		Phosphatidylethanolamine and/or lysine (combinations at 100/300, 200/200 and 300/100 ppm)	Phosphatidylethanol-amine and lysine showed a better protection to the oil than when each was added alone. The IP increased in the following sequence 100/300 < 200/200 < 300/100 ppm.	
		Phosphatidylcholine and/or lysine (combinations at 100/300, 200/200 and 300/100 ppm)	Phosphatidylcholine and lysine showed a better protection to the oil than when each was used alone. The IP increased in the following sequence 200/200 ≤ 300/100 < 100/300 ppm.	
		Phosphatidylcholine and/or phosphatidylethanolamine (combination at 100/300, 200/200 and 300/100 ppm)	Phosphatidylcholine & phosphatidylethanol-amine did not exhibit any synergism.	
Koprivnjak *et al.*, 2008	Filtered virgin OO, Rancimat, 120°C	Phospholipids (lecithin) (0, 2.5, 5, 7.5, 10 g/kg)	As the amount of lecithin increased, IP lengthened. The addition of lecithin also increased total tocopherols but decreased the α / γ tocopherol ratio.	
Lee and Choe, 2008	Tocopherol-stripped SFO; Water in oil emulsion consists of methylene chloride, n-butanol, $Na_2MoO_4.2H_2O$, sodium	Phosphatidylcholine (0, 250 and 1000 ppm)	Phosphatidylcholine extended the IP of the oil. Different phospholipids concentrations had the same effects.	

	dodecyl sulfate; rubrene (a singlet oxygen quencher); 25°C for 24 hr.			
Chen *et al.*, 2010	Stripped SBO, 25°C for 24 hr.	Dioleoylphoshatidyl-choline, dibutyrylphosphati-dylcholine, (each 0-1270 mmol/kg),	The association colloids formed by dioleoylphoshatidyl-choline and water were prooxidative, while those formed by dibutyrylphosphatidyl-choline were comparable to the control.	The structure of association colloids may influence lipid oxidation in bulk oils. Dioleoylphoshati-dylcholine and dibutyrylphosphati dylcholine have identical choline groups but different physical structure in oil. SAXS measurement revealed that dioleoylphoshati-dylcholine formed spherical structures while dibutyrylphosphati-dylcholine formed cylindrical structures.
Chen *et al.*, 2011b	Stripped SBO, 50°C	Dioleoylphoshatidyl-choline (1000 μM), α-tocopherol (10 and 100 μM), Trolox (10 and 100 μM)	Dioleoylphoshatidyl-choline formed reversed micelles in oil and shortened the IP. Dioleoylphoshatidyl-choline improved the activity of low α-tocopherol or Trolox concentrations (10 μM) but decreased the activity at high concentrations (100 μM).	
OH-containing carotenoids				
Haila *et al.*, 1996	TAG from low-erucic acid rapeseed oil, under dark at 40°C	Lutein (5, 20, 30, 40 ppm), lycopene (20 ppm), annatto (20, 30, 60 ppm as bixin), β-carotene (20 ppm), γ-tocopherol (10, 15 ppm), lutein + γ-tocopherol (20 + 15 ppm, 1:1 in molar ratio), or lutein + γ-tocopherol (20 + 10 ppm, 1.5:1 in molar ratio)	Lutein caused more LOOH. Lutein (20 ppm) + γ-tocopherol (15 ppm) were antioxidants. Lutein was consumed faster and slower (higher retention), without and with γ-tocopherol respectively. The consumption of γ-tocopherol was not affected by lutein. 30 and 60 ppm bixin (annatto) was significantly reduced LOOH levels.	Lutein was prooxidant both in the dark and light. When lutein is combined with tocopherol, they significantly increase oxidative stability. Lycopene and β-carotene were prooxidants.

Henry *et al.*, 1998	Purified safflower seed oil, OSI, 75°C for 24 hr., 85°C for 12 hr., 95°C for 5 hr.	Lycopene (35 µM), lutein (66 µM), 9-cis-β-carotene (54 µM) and all-trans-β-carotene (150 µM)	The orders of the rate of degradation were lycopene > 9- cis-β-carotene = all-trans-β-carotene > lutein.	Geometric configurations do not affect the decomposition rate, as in 9-cis and all-trans carotene. OSI in hours and lipid oxidation measurements (e.g. PV, TBARS) need to be performed to study the effects of carotenoids on safflower oil oxidation.
Subagio and Morita, 2001	Purified corn-oil TAG, paraffin, 40°C, dark	α-tocopherol, β-carotene, lutein (each at 5, 10 and 30 ppm and combination of 30 and 30 ppm)	Lutein increased the amount of LOOH. β-carotene + lutein caused more degradation of β-carotene. Lutein was more unstable than β-carotene in paraffin (medium similar to TAG).	The antioxidative effect of β-carotene was dose-dependent, at higher concentration it became prooxidant. When combined, β-carotene protected lutein, as β-carotene degraded more than lutein in oil. But in paraffin it is the opposite.
Kiokias and Gordon, 2003	Purified OO, oven, 60°C	β-carotene (1 g/L), annatto oil-soluble (bixin) (1 g/L) and annatto water-soluble (norbixin) (1 & 2 g/L), virgin olive oil polar extract (0.2 g/L), α- and γ-tocopherol, ascorbic acid and ascorbyl palmitate (each 0.1 mM)	Norbixin showed synergisms with ascorbic acid, ascorbyl palmitate and tocopherols, which were beyond the effect of phenolic antioxidants in oils and emulsions. These effects are better than that of β-carotene or β-carotene and polar extract.	The presence of polar carboxylic acid groups in the norbixin molecule may contribute to chelation of metal ions or other polar initiating species, thus retarding autoxidation of oil.
Becker *et al.*, 2007	Purified SFO; Rancimat (OSI), 100°C, 20 l/h	α-Tocopherol, rutin, astaxanthin, quercetin (each at 0.25, 0.5, 1.0 and 2.0 µmol antioxidant / g oil, and their combination of 1.0 + 1.0, 0.5 + 0.5 and 0.25 + 0.25 µmol / g)	The antioxidant ranking in bulk oil: quercetin > α-tocopherol >> astaxanthin = rutin.	Astaxanthin exerts antioxidant activity in bulk oil.
Zeb and Murkovic, 2011	Refined OO, Rancimat,	β-carotene, E-astaxanthin (300 ± 0.5 ppm) in olive oil	E-astaxanthin protected the oil from oxidation (reduced	9-Z-astaxanthin had a higher antioxidant effect among

	110°C, 1–14 h		epoxides) and inhibited β-carotene degradation.	astaxanthin. β-Carotene was prooxidant.
Amino acids				
Ahmad *et al.*, 1983b	Safflower oil, a mixture of sunflower and cottonseed oil, active oxygen method at 97.8°C	Cysteine, proline, tryptophan, methionine, glutamic acid, lysine, arginine (each at 0.01, 0.02, 0.04, 0.07, 0.10, 0.40, 0.70, 1.00%)	Cysteine and glutamic acid were prooxidants in the oil mix, and glutamic acid was prooxidants in the safflower oil. The highest protection activity in the safflower oil was due to methionine, proline, lysine, and cysteine. The highest protection activity in the mix was due to lysine, arginine, glutamic acid, methionine and hydroxyproline. However the amino acid protection activities were very low, low or medium.	Antioxidative activity of amino acids in such oils was low. It could be that they do not contribute as antioxidant by themselves, but requiring primary antioxidants.
Alaiz *et al.*, 1995	SBO, air in the dark at 60°C	N-(Carbobenzyloxy)-1(3)-[1'-(formylmethyl) hexyl]-L-histidine dihydrate (compound 1) formed from histidine and (E)-2-octenal (50, 100, 200 ppm), Z-histidine (50, 100, 200 ppm)	The order of stability: Compound 1 > Z-Hitidine > Control. The protection index of compound 1 and Z-histidine increased with concentration.	Reaction products of lipid oxidation (aldehydes) and amino acids exhibited an antioxidant property. Polymerization of these compounds produces melanoidin-like polymers causing changes in color and fluorescence.
Carlotti *et al.*, 1997	Linoleic acid in sodium dodecylsulphate micellar solutions (with ethylenediaminetetraacetic acid (EDTA) and azo-initiator	α-Tocopherol (1.0-6.0 x 10^{-6} M), α-tocopherol: 5.0 x 10^{-6} M and ascorbic acid: 0.4-1.0 x 10^{-4} M, α-tocopherol: 5.0 x 10^{-6} M and ascorbic acid: 0.5 x 10^{-4} M, L-tryptophan (8.5 x 10^{-5} M - 1.0 x 10^{-4} M), L-alanine (1.0 - 1.2 x 10^{-4} M), L-cysteine (8.5 x 10^{-4} M - 1.0 x 10^{-4}	Glutathione, L-tryptophan, L-alanine, L-cysteine, glycine prolonged the IP of micellar solution (containing 5.0 x 10^{-6} M α-tocopherol + 5.0 x 10^{-6} M vit C) at pH 5.0 and pH 7.0 (at 45°C). The synergistic action was particularly significant for L-	Amino acids exhibited antioxidative effects, either added alone or combination with other amino acids or α-tocopherol.

	added), pH 5.0 and 7.0, 45 and 56°C	M), glycine (1.0-1.5 x 10^{-4} M), and gluthathione reduced form (7.5-8.5 x 10^{-5} M)	cysteine, L-tryptophan and Glutathione.	
Hidalgo *et al.*, 2006	Refined OO, Rancimat, 110°C	Phosphatidylethanolamine, phosphatidylcholine, lysine, BHT (each 0, 100, 200, 300, 400 ppm and combination of phospholipids of 100, 200, or 300 ppm with amino acid of 100, 200 or 300 ppm)	Lysine (200 ppm or more) increased IP, which was superior to those of oil with phosphatidylethanolamine, phosphatidylcholine and BHT of the same concentration. 300 ppm phosphatidylethanolamine + 100 ppm Lysine caused 185% increase of IP compared to control, and when both were used alone. Phosphatidylcholine and lysine increased the IP.	Lysine and phosphatidylethanolamine or phosphatidylcholine exhibited synergism. The amino group of Lys reacted with oxidized lipids to form hydrophilic pyrroles, which are good for oil (polar paradox).
Papadopoulou and Roussis, 2008	Corn oil; 50, 120 and 180°C	N-acetyl cysteine and gluthathione (10, 20 and 40 mg/L), butylated hydroxyanisole (BHA) (200 mg/L)	N-acetyl cysteine and gluthathione reduced the oxidation prod. The antioxidative ranking were BHA > N-acetyl cysteine > gluthathione; except N-acetyl cysteine were more effective than BHA at 180°C.	N-acetyl cysteine and gluthathione are hydrophilic compounds, which have more affinities toward the air-oil interface in bulk oil, thus are more effective than lipophilic ones in bulk oil.
Hidalgo *et al.*, 2009	Stripped virgin OO, β-sitosterols added stripped virgin OO, Rancimat, 90°C, 10 L/h	Phosphatidylethanolamine (0, 100, 200, 300 and 400 ppm), phosphatidylcholine (0, 100, 200, 300 and 400 ppm), lysine (0, 100, 200, 300 and 400 ppm), β-sitosterols (1500 μg)	Phosphatidylethanolamine, phosphatidylcholine, lysine & their combinations cause a significantly higher antioxidative effects in β-sitosterol added virgin OO. The combined phospholipids gave a shorter IP but phospholipids + amino acids increased the IP, than when they were added alone.	Amino groups of lysine react with oxidized lipids to form hydrophilic pyrroles, which may contribute to the stabilization of oil.

Citric acid and ethylenediaminetetraacetic acid (EDTA)				
Hras *et al.*, 2000	Stripped SFO, oven, 60°C	Rosemary extract (0.02%), α-tocopherol(0.01%), ascorbyl palmitate (0.01%), citric acid (0.01%) and their combinations	Citric acid alone reduced peroxide value (PV) and anisidine value (AV), but the activity was lower than the extract and ascorbyl palmitate. The order of antioxidant activity: extract + ascorbyl palmitate > extract + citric acid > extract > extract + AT > control.	Citric acid is a chelating agent, which forms bonds between the metal and the carboxyl or hydroxyl groups of the citric acid molecule. Citric acid alone had an antioxidant role. The effect was greater when citric acid was combined with the extract.
Jaswir *et al.*, 2004	Cold pressed, unrefined antioxidant-free flaxseed oil, heated to 165 ± 5°C for 3.5 min., then 165°C for 6 min., and allowed to reach 60°C at room temperature.	Oleosin rosemary extract (0, 0.05, 0.1%), sage extract (0, 0.05, 0.1%), citric acid (0, 0.025, 0.05%)	Antioxidants (extracts and their combinations) significantly reduced PV, p-AV, FFA, color yellow, C18:1, C18:2 and C18:3, reduced some of absorbances at 232 and 268 nm, but increased C16:0 and C18:0.	Antioxidants added to the oil before frying were effectively retarding lipid oxidation and reducing oil hydrolysis during deep frying.
Wang *et al.*, 2005	Natural and randomized corn oil; OSI, 100°C	Citric acid (100, 200 ppm)	Randomized corn oil had a much lower OSI than natural corn oil. Citric acid (200 ppm) partially restored the OSI of randomized oil.	Citric acid protective effect is not related to chelation of transition metals.
Drusch *et al.*, 2008	Stripped refined fish oil, 20°C	α-tocopherol (100 ppm), δ-tocopherol (1000 ppm), ascorbyl palmitate (50, 500 ppm), lecithin (500, 2000 ppm), citric acid (100, 500 ppm)	Ascorbyl palmitate + citric acid (each at 500 ppm) reduced LOOH compared to the control and sample with 500 ppm ascorbyl palmitate + 200 ppm lecithin + (100 ppm or 500 ppm) citric acid.	Citric acid effects are due to its metal ions chelation ability, the action is greater when trace metal content is high and is trivial when trace metal content is low.
Yi *et al.*, 2011	Mixture of yellow palm olein/fish oil, mixture	Ascorbyl palmitate (200 and 500 ppm), citric acid (50 ppm), phosphatidylethanola	Citric acid and ascorbyl palmitate displayed an inhibiting effect on	Citric acid did not show synergisms with phospholipids.

	of red palm olein/fish oil, 30°C in the dark	mine (500 ppm), phosphatidycholine (500 ppm)	the formation of radicals, IP, PV and TBARS; but not the combinations of phospholipids, ascorbyl palmitate and citric acid).	
Wang *et al.*, 2005	Natural and randomized corn oil; OSI, 100°C	EDTA (100 ppm)	Randomized corn oil had a much lower OSI than natural corn oil. EDTA (100 ppm) completely restored the OSI of randomized oil.	The protective effect of EDTA is not related to chelation of transition metals.
Ascorbyl palmitate				
Frankel *et al.*, 1994	Stripped corn oil, 60°C in a shaker oven	α-Tocopherol, trolox, ascorbic acid,ascorbyl palmitate (each at 100 and 500 ppm)	α-Tocopherol and ascorbyl palmitate were more effective in o/w emulsion than in bulk oil. Ascorbic acid, Trolox and α-tocopherol + ascorbic acid or α-tocopherol + ascorbyl palmitate were more active in bulk oil, but ascorbic acid alone was better than α-tocopherol + ascorbic acid. LOOH and hexanal were measured.	Lipophilic antioxidants (α-tocopherol and ascorbyl palmitate) were more effective in o/w emulsions than bulk oil (or w/o emulsions). The opposite exists for hydrophilic antioxidants (ascorbic acid and Trolox). There is a strong synergism between α-tocopherol + ascorbyl palmitate and α-tocopherol + ascorbic acid, but α-tocopherol + ascorbic acid were not significantly better than ascorbic acid alone.
Gordon and Kourkimska, 1995	Rapeseed oil, heated at 80°C, deep fat frying, Rancimat 100°C	TBHQ (0.2 g/kg), lecithin (1 g/kg), ascorbyl palmitate (0.2 g/kg), rosemary extract (1 g/kg), BHT (0.2 g/kg), BHA (0.2 g/kg), D-δ-tocopherol (0.2 g/kg)	Rancimat results gave the order of antioxidant activity: TBHQ > lecithin > ascorbyl palmitate > rosemary extract > BHT, BHA, δ-tocopherol.	The effect on microenvironment modulation may sometimes be more important than hydrogen donation.
Hamilton *et al.*, 1998	Refined - deodorized Chilean anchovy fish oil, 20°C, free access	α-Tocopherol, δ-tocopherol, γ/δ-tocopherols (each was 0.006, 0.2, 1 and 2%), ascorbyl	Ascorbyl palmitate and lecithin alone gave a small improvement in oxidative stability (unlike tocopherols).	Ascorbyl palmitate promotes LOOH scissions. The function of lecithin is merely for solubilizing ascorbyl

	to air, RH 45%	palmitate (0.1 %), lecithin (0.5%).	Ascorbyl palmitate exhibited a pro-oxidant effect in the presence of 0.2 and 2% δ-tocopherol or γ/δ-tocopherol. Ascorbyl palmitate + lecithin and ascorbyl palmitate + 0.2 or 1% α-tocopherol (not at other level) displayed strong synergy.	palmitate (to partition in the o/w interface). The action of lecithin as an antioxidant is due to its phosphatidyl part which sequesters heavy metals, its ability to inhibit LOOH scission by ascorbyl palmitate and to react with free ascorbyl radicals.
Hras *et al.*, 2000	α-tocopherol free SFO, oven, 60°C	Rosemary extract (0.02%), α-tocopherol (0.01%), ascorbyl palmitate (0.01%), citric acid (0.01%) and their combinations	Ascorbyl palmitate significantly reduced PV and p-AV, compared to other additives and control, but the effects were lower than the extract. Combination of extract and ascorbyl palmitate resulted in the lowest PV and p-AV.	Rosemary extract contains phenolic diterpenes such as carnosic acid, carnosol, rosmanol; and other phenolic acid such as rosmarinic acid. Extract and tocopherol act as radical scavengers. Ascorbyl palmitate is an ascorbic acid derivative that is oil-soluble. Ascorbyl palmitate plays a role as an oxygen scavenger. Ascorbyl palmitate alone acted as an antioxidant but not when combined with an extract.
Frankel *et al.*, 2002	Algal oil containing 5.1-12.7% Eicosapentaenoic acid (EPA), 10.5-52.4% Docosahexaenoic acid (DHA), 11-19.37% α-tocopherol and carotenoids (577-2823 ppm); 40,	Ascorbyl palmitate (0.025%)	Ascorbyl palmitate caused an increase in the oxidative stability (PV and propanal) of the oil. Carotenoids at high concentration may have pro-oxidant effect by lowering the relative stability of certain algae oil.	Ascorbyl palmitate might have an antioxidant synergism with tocopherols.

	50, 60°C in a shaker oven			
Kiokias and Gordon, 2003	Tocopherol-stripped OO, oven, 60°C	β-carotene (1 g/L), annatto oil-soluble (bixin) (1 g/L) and annatto water-soluble (norbixin) (1 & 2 g/L), virgin OO polar extract (0.2 g/L), α-tocopherol (0.1 mM), γ-tocopherol (0.1 mM), ascorbic acid (0.1 mM), ascorbyl palmitate (0.1 mM)	Ascorbyl palmitate reduced PV of oil. Ascorbyl palmitate showed synergism with norbixin, which was beyond the effect of phenolic antioxidants, but lower than ascorbyl palmitate alone.	Ascorbyl palmitate significantly modulates the antioxidant activity of phenolic inhibitors.
Carelli *et al.*, 2005	SFO; stored at 30°C, Rancimat 130°C	Ascorbic acid, δ-tocopherol, ascorbyl palmitate, α-tocopherol, citric acid (each at 0, 100, 200, 400, 600, 800 ppm)	Ascorbic acid, ascorbyl palmitate, δ-tocopherol significantly increased IP. Ascorbic acid gave the most effect. Samples containing 100 ppm of each additive and control had similar PV, p-AV, and residual tocopherol. The polar compound showed an antioxidative synergism with ascorbic palmitate and δ-tocopherol.	There was an absence of linearity of OSI and concentration of ascorbic acid and ascorbyl palmitate, because they are consumed or participated in chain termination reactions and in one or more side reactions. α-tocopherol showed the greatest efficacy at < 700 ppm but not at higher levels because of its participation in side reactions.
	SFO; stored at 68°C; Rancimat 130°C	Ascorbic acid, δ-tocopherol, ascorbyl palmitate, α-tocopherol, citric acid (each at 0, 100, 200, 400, 600, 800 ppm)	Results of rancimat test of IP were the same as above. No significant differences in p-AV of all treatments. Antioxidant effectiveness in terms of PV was δ-tocopherol > ascorbyl palmitate > ascorbic acid > citric acid. Oxidized triglyceride monomers were lower from oil with δ-tocopherol, at a longer storage time.	At high temperature, oxygen has lower solubility in oil thus autoxidation rate is lower and becomes gradually replaced with polymerization, showed by formation of triglyceride dimer (at 68°C). Ascorbyl palmitate and δ-tocopherol protect the oil at higher temperatures.

	SFO; stored at 130°C; Rancimat 130°C	Ascorbic acid, δ-tocopherol, ascorbyl palmitate, α-tocopherol, citric acid (each at 0, 100, 200, 400, 600, 800 ppm)	Results of rancimat test of IP were the same as above. The p-AV showed antioxidant effects of ascorbyl palmitate and δ-tocopherol. Ascorbyl palmitate spared the highest tocopherol residual. Ascorbyl palmitate and δ-tocopherol gave the lowest polar compounds. δ-tocopherol showed lower oxidized triglyceride monomers.	Ascorbic acid could deteriorate at high temperatures, which lessens its antioxidant activity. Both oxidative and thermal degradation took place at 130°C, as shown by a significant increase in polar triglyceride and triglyceride dimer.
Olsen *et al.*, 2005	Refined and deodorized cod liver oil, 25°C in dark, Rancimat is done at 20 mL/min and 80°C	Tocopherol concentrate (800 ppm), ascorbyl palmitate (200 ppm)	The order of decreasing PV was tocopherl concentrate > control > tocopherol concentrate + ascorbyl palmitate. Tocopherol concentrate + ascorbyl palmitate caused a more grass/cucumber-like, than herring oil and paint flavors, which was more acceptable to consumers. Tocopherol conc. + ascorbyl palmitate inhibited the formation of the most volatile oxidation products, except hexanal groups. IP did not significantly change during storage, compared to initial IP.	Tocopherol + ascorbyl palmitate can be used to stabilize cod liver oil, in terms of odor & flavor, when the oil is stored in the dark at 25°C. Volatile compounds and IP did not give useful information about the resistance of the oils to autoxidation at 25°C.

Drusch *et al.*, 2008	Stripped refined fish oil, 20°C	α-tocopherol (100 ppm), δ-tocopherol (1000 ppm), ascorbyl palmitate (50, 500 ppm), lecithin (500, 2000 ppm)	Order of reduction of LOOH: 500 ppm ascorbyl palmitate > 2000 ppm lecithin > 500 ppm lecithin > 50 ppm ascorbyl palmitate. 500 ppm ascorbyl palmitate + 2000 ppm lecithin + (100 ppm α-tocopherol & 1000 ppm δ-tocopherol) gave the most effect in LOOH reduction, but increased propanal content. 500 ppm lecithin + others caused a lower effect than a single effect of lecithin.	The synergisms of ascorbyl palmitate, lecithin and α-tocopherol are owing to the antioxidative effect of lecithin (by regeneration of α-tocopherol from its oxidized radical or by interaction with ascorbyl radicals), a chelating effect of lecithin, or physical phenomena that might occured (better solubilization of antioxidants).
Karabulut, 2010	Butter oil TAG, oven, 60°C	Ascorbyl palmitate (5, 50, 100 and 200 ppm), α-tocopherol (10, 25 and 50 ppm), β-carotene (5, 10, 25, 50 ppm)	β-car. and ascorbyl palmitate had a higher oxidation rate (no IP) than that with α-tocopherol, but lower than the control; measured by PV and p-AV. There was synergism between ascorbyl palmitate + α-tocopherol, but not ascorbyl palmitate + β-car. (i.e. it had no effects).	The synergism of α-tocopherol and ascorbyl palmitate was due to α-tocopherol that was spared at the expense of ascorbyl palmitate during oxidation or ascorbyl palmitate is used to regenerate tocopherols.
Sorensen *et al.*, 2011	Water in oil emulsion containing 98% oil (fish oil and rapeseed oil, 1:1), 37°C in the dark	Ascorbic acid, ascorbyl palmitate, ascorbyl conjugated linoleic acid (ascorbyl conjugated linoleic acid), conjugated linoleic acid, each additive at 50, 100, 150, 200, 250 ppm; polyglycerol polyricinoleate (1%)	The IP as measured by LOOH showed that all additives acted as antioxidants. Propanal and hexanal concentration results were: ascorbyl palmitate > ascorbyl conjugated linoleic acid > ascorbic acid > conjugated linoleic acid.	Ascorbic acid, ascorbyl palmitate and ascorbyl conjugated linoleic acid owing to their antioxidative properties, mostly due to their ascorbyl group. Ascorbic acid is known as a radical scavenger of hydrophilic radicals and has a reducing power due to its ability to donate an electron to reactive free radicals.

Yi *et al.*, 2011	Mixture of yellow palm olein/fish oil, mixture of red palm olein/fish oil, 30° C in the dark, 100°C	Ascorbyl palmitate (200 and 500 ppm), citric acid (50 ppm), phosphatidylethanolamine (500 ppm), phosphatidylcholine (500 ppm)	Ascorbyl palmitate (200 and 500 ppm) alone or with citric acid displayed a pronounced inhibiting effect on the formation of radicals, lengthening IP, PV and TBARS in both oil. (Phosphatidylethanolamine or phosphatidylcholine) along with (ascorbyl palmitate or citric acid), the phospholipids exhibited a prooxidant effect.	Ascorbyl palmitate synergists with citric acid, but not with phosphatidylethanolamine and phosphatidylcholine.
Sterols				
Soupas *et al.*, 2004	Tripalmitin, 80°C for 1 - 8 week , 100°C for 3 - 48 hr., 140°C for 0.5 - 6 hr., 180°C for 0.5 - 6 hr.; purified rapeseed oil, 60°C for 1 - 7 days, 100°C for 3 - 48 hr., 140°C for 0.5 - 24 hr., 180°C for 0.5 - 6 hr.	Stigmasterol, sitostanol (each 1%)	Stigmasterol and sitostanol oxides (formed more) increased during all heat treatments in both mediums, except stigmasterol oxides during heating at 100°C for 0-6 hr. and sitostanol oxides during heating at 80°C for 0-4 weeks. At low T., the stigmasterol oxides had lower oxidation in tripalmitin than in the rapeseed oil. At all T., sitostanol was oxidized more at both matrices.	At high temperature, the unsaturated matrix is more readily oxidized than the stigmasterols, thus protecting the sterols; while the saturated matrix forces the stigmasterols to react. There is no relationship between sitostanol, matrix and temperatures, as sitostanol oxidized faster than both matrices.
Soupas *et al.*, 2006	Rapeseed oil, hydrogenated coconut oil, refined palm kernel oil, 4°C for 12 months.	Microcrystalline phytosterol suspensions contains of 77% sitosterol and 8% campesterol (18 and 30%).	30% phytosterol caused more phytosterol oxides than that of 18% phytosterol. 18% and 30% phytosterol, caused the sitosterol to be more oxidized in hydrogenated coconut oil and refined palm	The differences in the susceptibility of phytosterol oxidation in a different matrix are due to initial oxide contents in phytosterol preparation and lipid matrix. A higher level sitosterol is oxidized

			kernel oil, and rapeseed oil respectively. The phytosterol content at the beginning and after 12 months of cold storage did not change.	more readily than the unsaturated matrix (at 4°C).
Cercaci *et al.*, 2007	Corn oil, 55°C in the dark; phytosterol-oxide was made at 150°C for 2 h in an oven; hexadecane, 30°C for 24 h	Cholesterol, stigmasterol, β-sitosterol, 5-alfa-cholestane (1, 2, 3, 4, 5 mmol/kg hexadecane)	7-keto derivatives of phytosterols increased with time, the most being 7-ketositosterol. The ability of sterols to reduce interfacial tension was in the order: stigmasterol > cholesterol > β-sitosterol > 5α-cholestane.	Oxidation of phytosterols results in ketones, alcohols, epoxides and dienes. The 7-keto derivative is the major phytosterol oxidation product. Sterols have a planar rigid structure which causes them to pack together tightly, and with their ability to be surface active, they can concentrate at o/w interface of the microenvironment.
Winkler and Warner, 2008	SBO, high-oleic SFO, stripped SBO, stripped high-oleic SFO, Rancimat (OSI), 110°C	Mixed phytosterols consist of brassicasterol (3.8%), campesterol (26.9%), campestanol (0.6%), stigmasterol (17.2%), β-sitosterol (48.2%), sitostanol (1.1%), Δ5-avenasterol (1.3%), Δ7-stigmastenol (0.8%) (0.25, 0.5, 1 and 2.5%).	Phytosterols were prooxidants, by increasing dimers and polymerized triacylglycerol in stripped SBO and high-oleic SFO, but not after 4 hr. In stripped oil, phytosterol caused lower OSI.	At lower temperature and in a less unsaturated matrix (e.g. stripped high oleic SFO), phytosterols oxidized quicker than the matrix, it also causes higher dimers and polymerized TAG formation. But at a higher T and in a more unsaturated matrix (e.g. stripped SBO), Polyunsaturated fatty acids (PUFA) are oxidized preferentially over sterols to protect the sterols from oxidation.

Soupas *et al.*, 2006	Heat treated non-fat milks, long term storage at room T. and 4°C	Phytosteryl esters containing 45% sitosterol, 25% campesterol and 18% stigmasterol; phytostanyl esters containing 65% sitostanol and 33% campestanol (the sterol esters were added at 0.5%).	Phytosteryl esters oxidized more than phytostanyl esters. Temperature did not influence the oxidation effects of both antioxidants.	As phytosteryl esters oxidized more than phytostanyl esters, this could be because phytosteryl esters were used as antioxidants. Then they will protect the matrix more. The effects on oil matrix must be investigated.
Hidalgo *et al.*, 2009	Stripped virgin OO, β-sitosterols added stripped virgin OO, Rancimat, 90°C, 10 L/h	Phosphatidylethanolamine (0, 100, 200, 300 and 400 ppm), phosphatidylcholine (0, 100, 200, 300 and 400 ppm), lysine (0, 100, 200, 300 and 400 ppm), β-sitosterols (1500 μg)	Phosphatidylethanolamine, phosphatidylcholine, lysine and their combinations caused significantly higher antioxidative effects in phytosterol added OO, compared to stripped OO. Phosphatidylethanolamine / lysine and phosphatidylcholine / lysine increased the IP compared to when they are added alone, but not phosphatidylethanolamine / phosphatidylcholine. The effects were higher for lysine (200 ppm or more) and phosphatidylethanolamine / lysine (300/100 ppm) in stripped OO and phytosterol added OO.	There was a synergism among β-sitosterol and phosphatidylethanolamine; β-sitosterol and phosphatidylcholine; β-sitosterol and lysine; β-sitosterol and phosphatidylethanolamine + lysine; and β-sitosterol and phosphatidylcholine + lysine.
Salts				
Gurkov *et al.*, 2005	Water/air and water/oil (n-hexadecane and SBO) emulsions	Sodium dodecyl sulfate (SDS) (10^{-5}, 10^{-4}, 10^{-3} and 10^{-2}), NaCl (10 and 150 mM)	150 mM NaCl had a lower surface tension than 10 mM NaCl. SDS and NaCl caused a lower surface tension of SBO/water than in C16/water. The coverage of interface of the nanoemulsions at CMC was lower than 90%. The surface	Salt decreased CMC. The size and other parameters of nanoemulsions might need to be studied. The surface coverage did not depend on the type of fluid interface (air/water, oil/water with different

			coverage of SBO (with SDS and NaCl) was lower than that of hexadecane (with SDS and NaCl). There was an absence of saturation of the ionic surfactants at the CMC.	hydrocarbons) and salt concentration.
Calligaris and Nicoli, 2006	SBO; Rancimat, 120°C at 20 l/h	Potassium carbonate, potassium acetate, acetic acid, sodium acetate, sodium chloride, potassium chloride (all salts were added at 10% w/w)	Potassium carbonate and potassium acetate significantly reduced PV and hexanal. Others (acetic acid, sodium acetate, sodium chloride and potassium chloride) reduced hexanal and increased IP by Rancimat.	The antioxidant activity was attributed to the antichaotropic anionic species, of which could interact and form H bonds with LOOH.

2.5 The supramolecular chemistry of lipid oxidation

Bulk oils are not homogenous media, they contain oxidation products and many minor components. Microaggregates or micelles are formed in this heterophase system so that a minimum free energy state is achieved in the system. The driving force of micelles formation is the increase of entropy which occurs with the withdrawal of hydrophobic regions of surfactants from polar (water) regions and the subsequent disarrangement. When antioxidants and amphiphilic compounds are present, the hydrophilic head groups of these compounds are oriented and located towards the water phase of the microemulsions while their hydrophobic hydrocarbon tails stay in the oil phase (Smit *et al.*, 1991; Chaiyasit *et al.*, 2007; and Chen *et al.*, 2010).

The microemulsions are thermodynamically stable, single phase entities consisting of water, oil and surface active agents. Microemulsions occur in many different sizes, forms and have the ability to solubilize significant numbers of polar and non-polar compounds (Oldfield, 1994 and Garti, 2003). According to their

contents. Winsor systems classify microemulsions in to five kinds (Winsor, 1948; Surabhi *et al.*, 2010 and Mehta and Kaur, 2011):

-Winsor I: the lower (o/w) microemulsion phase is in balance with the upper excess oil, and consists of 2 phases,

-Winsor II: the upper (w/o) microemulsion phase is in balance with the lower excess water, also consists of 2 phases,

-Winsor III: the middle (o/w and w/o) bicontinuous microemulsion phase is in balance with the upper excess oil and lower excess water, and consists of 3 phases,

-Winsor IV: oil, water and surfactant are homogeneously mixed in one phase (isotropic), and

-Winsor V: two microemulsion's phases are immediately present, one is in contact with oil and another one is in contact with water.

Microemulsions that exist in bulk oils can be considered as a Winsor IV system (Chaiyasit *et al.*, 2007 and Chen *et al.*, 2011b), as bulk oils are not homogenous due to the presence of a small amount of water and minor components (Surabhi *et al.*, 2010). Microenvironments or so called association colloids, can exist in several types or forms, such as reversed micelles, lamellar, cylindrical aggregates *etc.* according to the kinds and levels of surface active compounds which are present in the oils (Chaiyasit *et al.*, 2007 and Chen *et al.*, 2010) (**Figure 2.4**). These microenvironments are kinetically unstable but thermodynamically stable, unlike emulsions which are kinetically stable but thermodynamically unstable. Finally, the phases will separate (Lawrence and Rees, 2000).

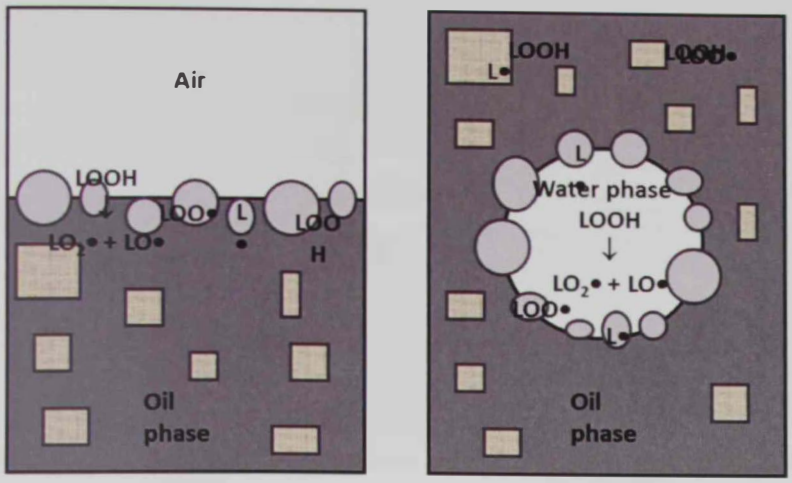
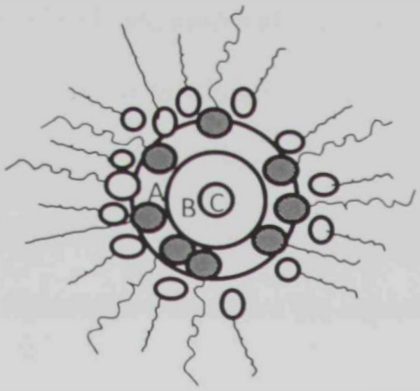


Figure 2.4. The location of hydrophilic ○ and hydrophobic ▭ antioxidants at the oil-air (left, Frankel *et al*., 1994) and oil-water (right, Chaiyasit *et al*., 2007) interfaces of bulk oils, with lipid radical, hydroperoxides and water

Figure 2.4 illustrates that micro- or nano-emulsions of association colloids (Chaiyasit *et al*., 2007) would better explain lipid oxidation reactions in bulk oils than oil/air partitioning (Frankel *et al*., 1994). In bulk lipids, micro- or nano-emulsions have been identified to contain several regions or layers: the core of micelles which consist of minuscule amounts of water and other polar compounds (such as salts and acids), a depletion layer which is a layer of water of a few molecular diameters in thickness, where surfactants cannot be found and is located between the core and monolayer; and a monolayer or interface which consists of only surfactant compounds (Smit *et al*., 1991) (**Figure 2.5**).



Surfactant: dark grey
Co-surfactant: white

Figure 2.5. A reversed micelle stabilized by surfactants and co-surfactants. Symbols: A=monolayer of surfactants, B=depletion layer and C=core (water)

As bulk oils contain small amount of water, it is worth discussing the evolution and importance of water that is present in the oils. Water activity, the measure of how water can influence a reaction, can also affect lipid oxidation rates due to its correlation with metal reactivity and hydroperoxide stability (Frankel, 1998). The rate of lipid oxidation in food products is the lowest at water activity of 0.2-0.4 and increases with higher water activity (El-Shattory *et al.*, 2003 and Chaiyasit *et al.*, 2007). When water activity is increased, oil viscosity is reduced and metals are mobilized and react with unsaturated fats and oxidation becomes faster (El-Shattory *et al.*, 2003). Increased water content may also increase the rate of oxidation, as there is a possibility that water can hydrolyze triacylglycerols (TAG) and produce mono-, diacylglycerols and free fatty acids which were found as prooxidants in many studies (**Table 2.3**). Different types and levels of water and surface active agents produce different shapes of association colloids and in turn will differently influence lipid oxidation (Chen and Terentjev, 2010 and Chen *et al.*, 2011b). In the case of phospholipids, they enhance the activity of α-tocopherol only when a small amount of water is present with catalysis by water-soluble radical generators (Koga and Terao, 1995). This implies that water in

microenvironments affects lipid oxidation and thus needs to be controlled (Nicholson, 2005). In addition, an increase in the length of the acyl moieties of phospholipids causes a positive or lengthening effect to the IP (Koga and Terao, 1995 and Khan and Shahidi, 2000). The fatty acids composition and kinds of functional groups in the phosphate groups of phospholipids also affect the oxidation in a different way (Chen *et al.*, 2011b).

At low levels, around 230-240 ppm, water does not exert effects on oxidation, even though oil has a high interfacial tension and much oxidation (Chaiyasit *et al.*, 2008). When water content increases up to 1000 ppm, this water also does not cause a significant effect on oxidation as the water is bound to polar compounds and stays in the multilayer of the association colloids (Chen *et al.*, 2011b). Water in refined oils, starting approximately at 300 ppm comes from water that is found in oilseeds and residues of water from neutralization and degumming processes (Chaiyasit *et al.*, 2007 and Chen *et al.*, 2010 and 2011b). Water content in oils increases throughout oxidation, as water is formed by monomolecular and bimolecular decomposition of hydroperoxides during the propagation stage (discussed in the next Chapter).

As mentioned earlier, bulk oils contain varieties of surface active compounds such as minor components (for instance FFA, MAG and phospholipids) and lipid oxidation products (such as hydroperoxides, aldehydes, ketones and epoxides). These amphiphiles with a small amount of water can form microemulsions or association colloids that can affect oxidation (Chaiyasit *et al.*, 2007 and 2008; Kasaikina *et al.*, 2008; and Chen *et al.*, 2010 and 2011b). The stabilization role of the minor components on the oils is exhibited in original corn oil which has lower interfacial tension (20.1 ± 0.09 mN/m) compared to the stripped one (31.5 ± 0.68 mN/m), respectively. This indicates that minor components lower the interfacial tension and overall energy of the

system (Lawrence and Rees, 2000; Chaiyasit *et al.*, 2007 and 2008). The reversed micelles or association colloids in bulk oils were measured to be in the range of 1-500 nm (Kasaikina *et al.*, 2008 and 2010). Refining of vegetable oils removes detrimental minor components (such as free fatty acids which cause foaming and reduce smoke point of the oils, and chlorophyll which plays a role as photosensitizer), but also has some undesirable effects due to the removals of antioxidants (i.e. tocopherols) and emulsifiers (Chaiyasit *et al.*, 2007 and 2008 and Chen *et al.*, 2011b). As a result, the rate of lipid oxidation in the refined oils is higher than that of the unrefined ones. However, some traces of these compounds exist in the bulk oils after refining, and can still affect the oxidation of the oils (Chen *et al.*, 2011b). The structures of these microemulsions can be investigated by using technologies such as cryo-transmission electron microscopy (cryo-TEM), dynamic light scattering (DLS), small-angle neutron scattering (SANS), wide-angle X-ray scattering (WAXS) and small-angle X-ray scattering (SAXS) (Gupta *et al.*, 2001).

Microemulsions can assume several forms. The kinds of microemulsions and the effects they might have in the bulk oils are likely to depend on the antioxidant's and additives's hydrophilic lipophilic balance (HLB). The empirical definition of HLB relates to its indication to the solubility of an antioxidant in the lipid medium. The HLB concept was proposed by Griffin (1954), and is based on the relative parts of the hydrophilic and hydrophobic groups of the (surfactant or antioxidant) molecules. Griffin (1954) proposed the HLB empirical equation for non-ionic alklyl polyglycol ethers (C_iE_j) as follows:

$$HLB = (Ej\ wt\% + OH\ wt\%) / 5$$

Where, E_j wt% is the weight of ethylene oxide in % and OH wt% is the weight of hydroxide group in %.

Davies (1957) then explained the HLB further and proposed a more general formula by introducing constants for the hydrophilic and hydrophobic groups:

$$HLB = [(n_H \times H) - (n_L \times L)]$$

Where n_H is the number of hydrophilic groups in the molecule, n_L is the number of lipophilic groups in the molecule, H is the constant for hydrophilic groups, and L is the constant for lipophilic groups.

Considering the HLB of a compound, water in oil (w/o) microemulsions are formed when HLB is less than 10, thus surfactants or compounds with low HLB (3-6) have more tendencies to form w/o emulsions (Oldfield, 1994). On the other hand, oil in water (o/w) microemulsions are formed when HLB is more than 10, hence additives with higher HLB (such as 8-1) tend to stabilize this o/w emulsions (Israelachvilli *et al.*, 1976 and Mitchel and Ninham, 1981). Minor components with low HLBs such as free fatty acids (1.0), mono- (3.4-3.8) and diacylglycerols (1.8), prefer to form and enhance reversed micelles or w/o microemulsions (Chaiyaisit *et al.*, 2007). Whereas, a phospholipid with intermediate HLB of around 8.0, is able to form many kinds of association colloids, such as spherical reversed micelles with small amounts of water (<0.3%) that are present in bulk oils, and lamellar structures with the presence of other surfactants (Chaiyasit *et al.*, 2007 and Chen *et al.*, 2010).

Besides HLB, another factor namely a surfactant packing parameter (Sp) also influences the kinds of emulsions and association colloids formed and stabilized in the medium (Israelachvilli, 1994 and Surabhi *et al.*, 2010). The Sp concept uses a more quantitative approach by considering the geometry and curvature preference of a surfactant molecule, and the concept is particularly used when a co-surfactant is present (Oldfield, 1994 and Surabhi *et al.*, 2010). A co-surfactant can be defined as a substance that can improve or synergize the physical dissolution (increase the

solubility) of surfactants in organic solvents, and thus facilitates the formation of reversed micelles and helps to stabilize the microemulsions (Krei *et al.*, 1995 and Mathew and Juang, 2007). The Sp concept was introduced by Mitchell and Ninham in 1981 (Mitchell and Ninham, 1981) and can be calculated as follows:

$$Sp = v / (a_o \times l_c)$$

Where, v is the volume occupied by one tail,

a_o is the interfacial surface area occupied by one molecule, and

l_c is the length of surfactant (measured radially from the interface)

A higher Sp value of more than 1 is needed to stabilize w/o microemulsions, whereas a smaller Sp value is preferred for the formation of o/w emulsions.

Related to the focus on physical effects, in some cases co-surfactants coexisted or were added with surfactants in the system. Co-surfactants that are widely used in the pharmaceutics include short-chain alcohols C3-C6, medium chain length alcohols C6-C12, glycerol, sorbitol, geraniol, and fatty acid sucrose esters (Lawrence and Rees, 2000 and Surabhi *et al.*, 2010).

The arrangement of co-surfactants in the micelles is not quite clear. However, co-surfactants have the ability to partition between the oil and water phase, thus it has been advocated that they are also located in the interface (Hait and Moulik, 2002) or in the oil phase in between the surfactant molecules and form complexes with the surfactants (Mathew and Juang, 2007) (**Figure 2.5**). Co-surfactants are weak amphiphile molecules (Kahlweit *et al.*, 1991), and contribute by lowering the electrostatic repulsion between the surfactant head groups. As a result the following occurs: weak hydrophobic interactions between surfactant tails, increase of the volume and mobility of surfactant tails (v), and reduce the interfacial surface area (a_o) which finally lead to a modulated packing of surfactants in the micelles (Leodidis and Hatton,

1989; Karpe and Ruckenstein, 1991; and Lawrence and Rees, 2000). The effects of co-surfactants also depend on their size and chain length (Graciaa *et al.*, 1993a and b). Their contributions to the micelles include keeping water inside the micelles and minimizing their size (Mathew and Juang, 2007). System conditions and molecular geometry of the molecular aggregates in o/w and w/o emulsions in the presences of surfactants and/or co-surfactants are illustrated in **Figure 2.5**. The contribution of molecules which could have co-surfactant properties (such as citric acid and amino acids) to lipid oxidation has not been explored, however the mechanisms of such compounds might be explained as synergists (Ahmad *et al.*, 1983a). Furthermore, the antioxidant mechanisms of some secondary antioxidants and minor components are not well understood. These compounds could modulate the physical aspects by affecting or stabilizing microemulsions, the oxidation site. In this way they could be considered as synergists. The historical development in the knowledge of the physical factors that influence lipid oxidation is presented in **Table 2.1**.

2.6 Hydrophilic-lipophilic balance and the cut-off effect of antioxidants

A cut-off effect of the polarity of antioxidants was observed in studies of o/w emulsified systems. In one of these studies, the antioxidant capacity (tested by conjugated autoxidizable triene assay) of homologous series of chlorogenic acid esters increased as lipophilicity increased (that was achieved by increasing alkyl chain length). However only until chain length of C12 did the antioxidant capacity also increase. Then from C16 there was a sudden decrease of the antioxidant capacity (Laguerre *et al.*, 2009). In another study, the antioxidant capacity (as analyzed by surfactant effectiveness) of hydroxytyrosol fatty acid esters increased with the increase of lipophilicity until hydroxytyrosol decanoate (C12). However, further increases of

chain length caused a sudden cut in the surfactant effectiveness (Lucas *et al.*, 2010). This cut-off effect of antioxidant effectiveness is related to the molecular size of antioxidants and their mobility, wherein bulky compounds (i.e. compounds with long alkyl chain) have lower mobility due to steric hindrance and lower diffusability in the oxidation sites (Takahashi *et al.*, 1992 and Stockmann *et al.*, 2000).

Besides the cut-off effects, there is a nonlinearity of the general role described by the polar paradox. The rule that polar (hydrophilic) antioxidants are more effective in bulk oils and the nonpolar (lipophilic) antioxidants are more protective to emulsions, is not always demonstrated. Shahidi and Zhong (2011) observed that as lipophilicity of EGCG and its esters increases, their antioxidant activity was exhibited in bulk oils at low levels, but as hydrophilicity increases the antioxidant activity was at higher levels in bulk oils. This indicates the possibility that (polar paradox) hydrophilic antioxidants are applicable only at certain higher concentration (to reach its critical concentration) in order to protect bulk oils, which also means that at this higher concentration the interfacial phenomena is more important than the solubility effects (and the converse at lower concentration of the antioxidants). This nonlinearity effect was also found in other pairs of antioxidants, such as Trolox / α-tocopherol, ascorbic acid / ascorbyl palmitate, and gallic acid / propyl gallate (Shahidi and Zhong, 2011).

Therefore, HLB is not the only factor affecting the rule of polar paradox, other factors such as antioxidant's molecular size, configuration and their concentrations also govern antioxidant effectiveness. Therefore the polar paradox can only explain the antioxidant effectiveness in a system from a general understanding and is not always verified. The above discussion also suggests that "pure chemical thinking" is not sufficient (lacking of explanations), a greater understanding of supramolecular

chemistry is needed in order to fully investigate the antioxidant's effectiveness in a system.

2.7 Various factors influencing the supramolecular chemistry

It has been well studied that there are other factors which influence antioxidant activity in bulk oils. Besides the degree of the unsaturation of the fatty acid, other factors include diffusion of oxygen, temperature and light (Kamal-Eldin and Appelqvist, 1996 and Choe and Min, 2006), interaction of antioxidants with prooxidants and amphiphilic minor components (e.g. phospholipids, MAG, FFA (Chen *et al.*, 2010), the presence of other surface active compounds (their HLB, nature and ratio) (Garti, 2003), lipid composition-structure-position (Winkler and Warner, 2008 and Belhaj *et al.*, 2010), other food components (Medina *et al.*, 2010), physical structures (Chen *et al.*, 2011b), pH (Kellerby *et al.*, 2006 and Chen *et al.*, 2012b) structures of microenvironments, interfacial characteristics (Mei *et al.*, 1998b; Mancuso *et al.*, 1999; and McClements and Decker, 2000), stability of EPA and DHA (Shahidi and Zhong, 2010), and position of fatty acids on glycerol backbone (Endo *et al.*, 1997a). A global conceptual framework however, has never been agreed upon because of the inconsistencies and paradoxical outcomes in some studies. With regards to the importance of microenvironments and supramolecular chemistry in bulk oils, it is clear that these factors or effects should be revisited and investigated. The new paradigm of the supramolecular chemistry in bulk oils includes the recognition and consideration of molecular shapes and the relative positions of hydrophilic and lipophilic regions. **Figure 2.6** illustrates these features belonging to the unsaturated fatty acids and minor lipid components. In an addition, understanding the role of synergists with high topological polar surface to volume ratio (e.g. phytic acid, EDTA

and citric acid) (**Figure 2.7**) becomes important to wholly understand the influence of the supramolecular orientation of additives and native molecular species on the rate of lipid oxidation.

Many association colloids are sensitive to their structural details in the relations to lipid oxidation (Chaiyasit *et al.*, 2008). For example, the effects of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC_4PC) when combined with Trolox on the rate of oxidation of stripped soybean, are related to the formed micelle's size and shape (Fanun, 2009 and Chen *et al.*, 2011b). DOPC formed reversed micelles and brought a prooxidative effect in stripped soybean oil while DC_4PC produced cylindrical structures (not reversed micelles) and had no oxidation effects to the oil (Chen *et al.*, 2010, 2011a and b). These studies suggested that surfactants can also influence antioxidant's effects.

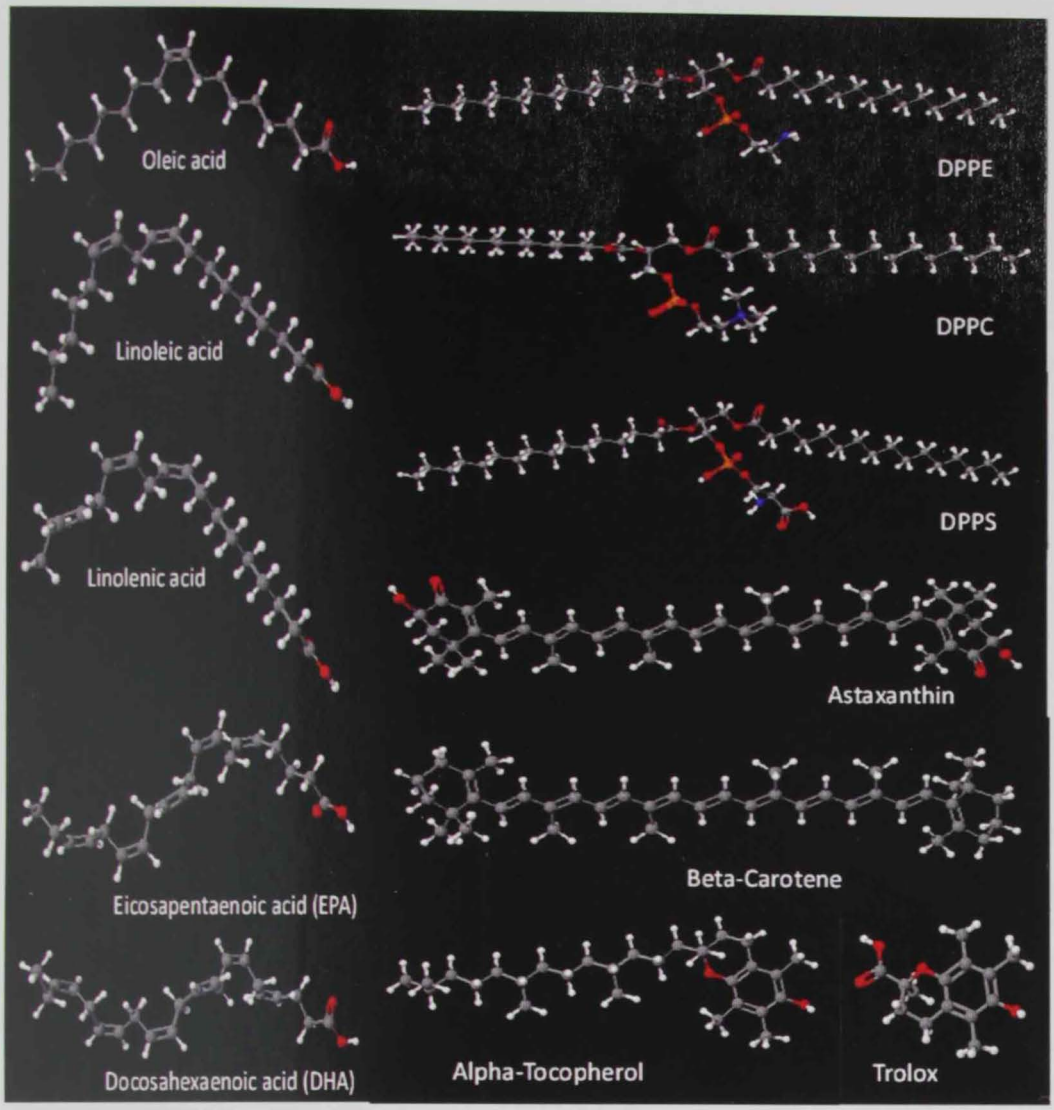


Figure 2.6. Space filling models of unsaturated fatty acids and selected minor lipid components and antioxidants. Structures were drawn with permission from www.molecular-networks.com/online_demos.

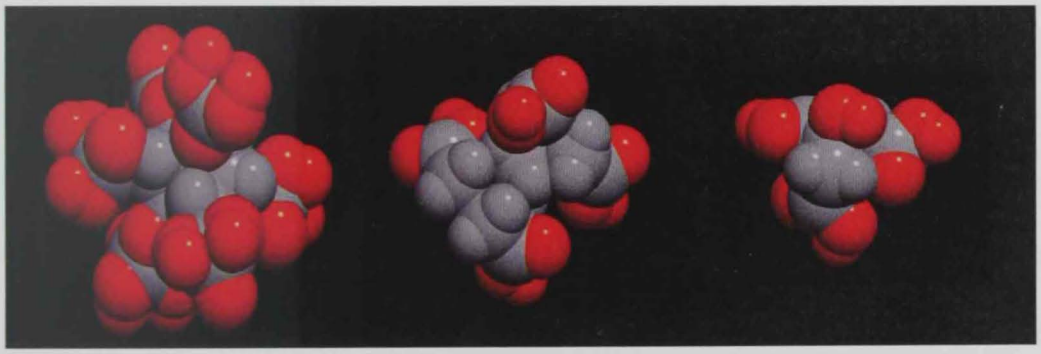


Figure 2.7. Chemical space filling models of phytic acid, EDTA, and citric acid antioxidant synergists showing their topological polar surfaces. These retarders may

exert their synergistic effects through interface modulation in addition to metal chelation. Structures were drawn with permission from www.molinspiration.com.

The reasons could be due to the phospholipids aliphatic chains, which m odulates the size and shape of micelles (Fanun, 2009) and the negative charged surface of the reversed micelles formed by DOPC that attracts metals (such as iron) and hydroperoxides to locate at the oil/water interface which further enhances the decomposition of hydroperoxides (Chen *et al.*, 2011b). Similarly, phosphatidylcholine (1,2-dibutyl-sn-glycero-3-phosphocholine) did not form reversed micelles and was not prooxidative in the oils (Chen *et al.*, 2012a). In addition, phospholipids zwitterionic effects (Lawrence and Rees, 2000) can cause charge-related interactions (Chen *et al.*, 2011b) to the micelles with transition metals.

Interfacial characteristics due to ionic surfactants can cause a significant impact on oxidation. Anionic surfactants, such as sodium dodecylsulfate (SDS), and sodium dioctyl sulfosuccinate or (AOT) can arrange themselves to large micelles (Kortenska *et al.*, 2002) and create interfaces with negative charges that have affinities for metal binding, thus increasing the lipid oxidation in o/w (Chaiyasit *et al.*, 2007 and 2008). However, such anionic surfactants cause no significant effects on the oxidation of lipophilic substrates, such as limonene (Kasaikina *et al.*, 2010). On the other hand, cationic surfactants, such as cetyltrimethylammonium bromide (CTAB) and quaternary ammonium alkyl salts, generate numerous amounts of very small micelles (Kasaikina *et al.*, 2008 and 2010; and Chen *et al.*, 2011b) and accelerate oxidation in bulk oil and limonene, but lower the oxidation of o/w (Kasaikina *et al.*, 2008 and Surabhi *et al.*, 2010). In order to mitigate these effects, co-surfactants are often added to increase the micellar size and stability (Pessoa and Vitola, 1988). In addition, cationic surfactants were found to influence oxidation of bulk oils, at the initiation

phase, but not at the propagation and termination phases (Kaşaikina *et al.*, 2008). Nonionic surfactants (e.g. polyoxylene lauryl ether (Brij 35), sorbitan oleate (Span 80), sugar surfactants such as alkyl glucosides and sucrose fatty acid esters, mono- and diacylglycerol-, medium chain triacylglycerides, fatty acid esters such as isopropyl myristate) were reported to improve the oxidative stability of emulsions (Lawrence and Rees, 2000; Oehlke *et al.*, 2010 and Sun *et al.*, 2011), however these findings are not averred as many studies had different outcomes (Mei *et al.*, 1999 and Sun *et al.*, 2011). These findings may give possibilities for molecular modifications of surfactants, such as by esterifying free –OH group(s) MAG or DAG with other molecules such as citric acids.

The position of the double bonds of unsaturated fatty acids also influences oxidation and was reported in colloidal dispersion systems (Miyashita *et al.*, 1995). When the double bond is located near to the methyl end of the molecule, it causes increases in stability (McClements and Decker, 2000). *Trans* fatty acids are more stable than *cis* fatty acids, due to the former's molecules having a straight configuration, hence the molecules are tightly packed and have higher melting points (Talbot, 2013). A pH of a system also affects the lipid oxidation. A study of oil/water emulsions showed that when the lipid droplets were coated with proteins, at pH < pI, these droplets had a cationic interface and hence transition metals were repelled and oxidation was retarded (Kellerby *et al.*, 2006 and Chen *et al.*, 2012b). Whereas a higher pH resulted in negative effects on lipid oxidation, as more iron is precipitated onto an emulsion surface and reacts with the unsaturated lipids (Mancuso *et al.*, 1999 and Sun *et al.*, 2011).

Special considerations are required when discussing the oxidation of fish oils, due to the high degree of unsaturation and the highly bent structure of their very long

chain omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Cho *et al.*, 1987). The positions of the unsaturated fatty acids in the triacylglycerols backbone also contribute to the rate of oxidation in fish oils (Miyashita and Takagi, 1988). Mammalian oils, for instance seal and whale oils, are more stable against oxidation compared to fish oils, because their fatty acids (EPA and DHA) are located at sn-1 and sn-3 at the triacylglycerol backbone, while those of fish oils are located mainly at the sn-2 positions. (Kimoto *et al.*, 1994; Endo *et al.*, 1997a and b; and Wanasundara and Shahidi, 1997). It was advocated in another study that the stability of triacylglycerols is compromised when EPA is highly located at sn-2 of the triacylglycerol molecules (Endo *et al.*, 1997c). However, in the case of soybean oils, the locations of its unsaturated fatty acids at sn-2 position help to improve the oxidative stability of the oils (Endo *et al.*, 1997a). This suggests that the substrate or the lipid system also influence oxidation, besides the type and location of the unsaturated fatty acids. The bent structure of fish/mammalian fatty acids causes different orientations in the interfacial space and of molecular distributions. In dispersed systems with smaller particles (i.e. bulk lipids with many heterogeneous minor components), it is imperative to give attention to the nanoemulsions structure, sizes and dispersion phase. More stability was exhibited in systems contining smaller particles (Adachi *et al.*, 2009). Some types of fish lipids, such as fish roe lipids, contains α-tocopherol and phospholipids (synergist) which cause the oils to be more stable, despite their high amounts of EPA and DHA, compared to other kinds of fish oils. Similar synergisms were also exhibited in perilla oil. Thus phospholipids act synergistically with α-tocopherol in protecting the oil (Kashima *et al.*, 1991; Hara *et al.*, 1992; King *et al.*, 1992a and b; and Shahidi and Zhong, 2010). The role of primary antioxidants and

synergists in the initial phase of lipid oxidation is to stabilize micelles (**Figure 2.8**) as well as to scavenge radicals.

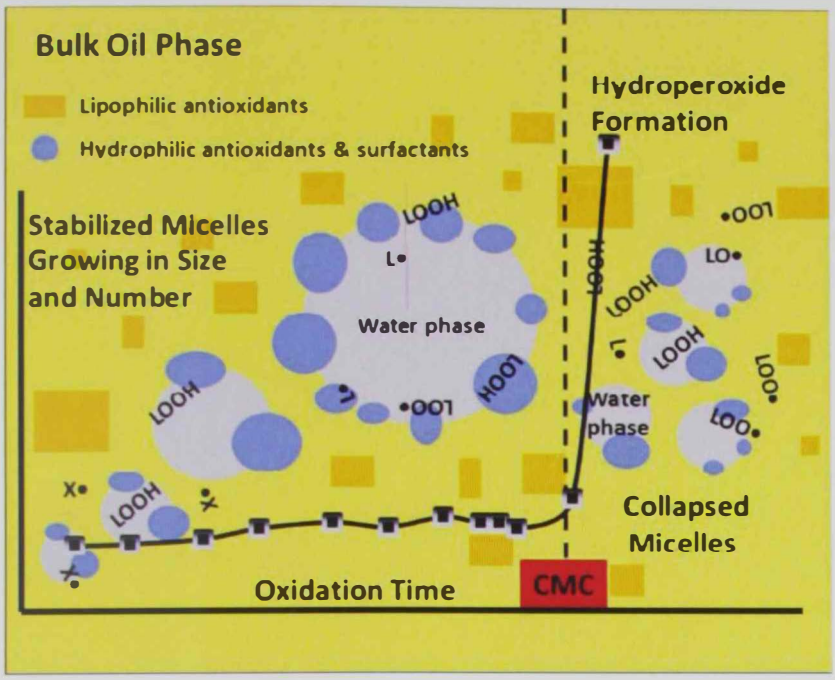


Figure 2.8. Scheme of the stabilization of reversed micelled by antioxidants and synergists, showing the transition from the initiation phase to the propagation phase. This transition occurs when the micelles reach their critical micelle concentration (CMC).

CHAPTER THREE

Changes in water content and micelle size during the oxidation of sunflower and canola oils

3.1 Introduction

Polyunsaturated fatty acids (PUFA) with bisallylic hydrogens, are susceptible to oxidative reactions leading to an undesirable flavor, loss of nutrients, and shortened shelf-life of products containing them (Chan, 1987). Lipid oxidation occurs in three phases; initiation (or induction), propagation and termination (Schaich, 2005). The initial phase witnesses slow oxygen incorporation and buildup of hydroperoxides and is marked by the induction period (IP), a critical point at the end of this phase and the start of the propagation phase that is driven by self-catalysis by hydroperoxyl radicals.

During past decades, the understanding of lipid oxidation was mainly focused on the free radical reactions involved in the propagation reactions. These was a lack of the minimal, but yet critical, events which take place during the initial phase. Lipid oxidation during the induction period has long been monitored by following development. of hydroperoxides (by means of the peroxide value or conjugated dienes). This was indeed due to the difficulty in measuring minute physicochemical alterations during the initial phase.

Gradually, evidence has accumulated to suggest that the criticality in the transition from the initiation phase to the propagation phase is governed by the attainment of a critical micelle concentration (CMC) of hydroperoxides and other amphiphilic compounds, such as mono- and diacylglycerols, phospholipids, free fatty acids, sterols, and trace amounts of water (Brimberg, 1993a; Koga and Terao and Brimberg and Kamal-Eldin, 2003a). Amphiphiles with low hydrophilic lipophilic balance (HLB in the range 1-4) form water in oil emulsions in bulk oils (Chaiyasit *et al.*, 2007 and Chen *et al.*, 2011b). Oxidation products formed during oxidation, including hydroperoxides, alcohols, aldehydes and ketones, are amphiphilic in nature and they contribute to micelle formation in bulk oils. These surface active molecules arrange together into supra molecular structures of higher orders and form

microenvironments in bulk oils including lamellar structures and reverse micelles (**Chapter 2**). Initially, the nano- and micro- reverse micelles in bulk oils are stabilized by existing antioxidants but as the concentration of hydroperoxides and other amphiphiles increases, the micelles grow in number and size and then collapse. After this critical point, oxidation enters the propagation phase where oxidation involves free radical reactions. By positioning their polar head groups at the interface of the micelles and their nonpolar tails at the oil phase, antioxidants in bulk oils lower the interfacial tensions, stabilize the microemicelles, and scavenge radicals at the interfaces leading to prolonged induction periods (Smit *et al.*, 1991; Brimberg, 1993a; Brimberg and Kamal-Eldin, 2003b; Decker *et al.*, 2005; Chaiyasit *et al.*, 2007; Kasaikina *et al.*, 2008; Sun-Waterhouse *et al.*, 2011).

Vegetable oils contain small amounts of water (in the range 0.02-0.05%) coming from the water in oilseeds and/or the wash water in refining processes with the exception of virgin olive oil, which contains 0.09% of water (Chaiyasit *et al.*, 2007; Chen *et al.*, 2010 and 2011). The amount of water increases during oxidation as a result of the mono- and bimolecular decomposition reactions of lipid hydroperoxides (ROOH),

$ROOH + RH \rightarrow RO\bullet + R\bullet + H_2O$ (Yanishlieva *et al.*, 2002)

$2\ RO_2H \rightarrow RO_2\bullet + RO\bullet + H_2O$ (Schaich, 2005)

And this evolution of water content is also expected to contribute to the length of the oxidation lag phase.

The aims of this research were to follow the oxidation of two vegetable oils, namely sunflower and canola in their unpurified and purified forms, with respect to hydroperoxide formation, loss of tocopherols and unsaturated fatty acids, and evolution of water and micellization during the initial phase of lipid oxidation.

3.2 Materials and methods

Oils and their purification

Two commercial vegetable oils, sunflower and canola, were purchased from the market and purified according to the procedure described by Fuster *et al.* (1998). Briefly, 250 g Al_2O_3 (activated at 100°C for 8 hours and then at 200°C for 12 hours) were suspended in 200 ml n-hexane and packed in a glass column (40 cm length x 3 cm inner diameter) plugged with glass wool. The oils (100 ml dissolved in 100 ml n-hexane) were passed through the column and the purified oils were collected in an amber colored bottle and kept in hexane in the freezer until the day of use. Directly before incubation, hexane was evaporated from the purified oils.

Oxidation experiments

For the unpurified sunflower and canola oils, samples (75 g) were placed in 400 and 800 ml beakers (10 beakers for each size) and were oxidized at 40°C for sunflower oil and 50°C for canola oil. At each day of analysis, one beaker from each treatment was taken and *ca* 1.3 g oil was sampled from each beaker and the beaker was placed back in the oven. Other beakers were used for the other days of analysis. Samples were analyzed for 25 days, by which the oil content in each beaker was not less than 70 g.

For the purified oils, samples (20 g) were placed in 400 ml and 800 ml beakers (6 beakers each) and the purified sunflower and canola oils were oxidized at 40°C and 50°C, respectively. At each day of analysis, one beaker from each treatment was taken and *ca* 1.3 g oil was sampled from each beaker and the beaker was placed back in the oven. Other beakers were used for the other days of analysis. Samples were analyzed

for 7 days for purified sunflower oils and 3 days for purified canola oils by which the oil content in each beaker was not less than 17 g.

Monitoring of lipid oxidation:

Fatty acid methyl esters (FAMEs) were prepared according to the AOAC Method 969.33 (AOAC, 2007) as described in Kowalski (2007). The fatty acid composition was analyzed by gas chromatography (Young YL Instrument 6500 GC System, Gyeonggi-do, South Korea) on SP-2380 columns (30 m, 0.25 mm ID x 0.20 μm film, Sigma Aldrich, St. Louis, MO) heated at 50°C for 2 min and then to 250°C at 4°C/min and held at 250°C for 15 min using helium as the carrier gas (20 cm/sec). The injector and detector temperatures were 150°C and 260°C, respectively. Peroxide value (PV) was measured according to International IDF Standard method 74A:1991, as described by Shantha and Decker (1994). The induction period (IP) of the oils was determined by a tangent method from the lines that pass through most of the points from both sides.

Residual tocopherols were analyzed by HPLC (Waters 2695 Separations Module, Alliance, Milford, MA) according to Kamal-Eldin *et al.* (2000). The column was 25 cm x 4.6 mm (5 μm) normal phase silica (Supercosil LC-Si HPLC Column, Sigma Aldrich, St. Louis, MO), the mobile phase was hexane:2-propanol (99:1, v/v), and the flow rate was 1.0 mL/min. Peaks were detected with Waters 2475 Multi λ fluorescence detector at an excitation wavelength of 294 nm and an emission wavelength of 326 nm. External calibration curves for α- and γ-tocopherols (Tocopherol set - Calbiochem, ≥95% purity by HPLC, prod. no. 613424, Merck, Darmstadt, Germany) were utilized to quantitatively determine the tocopherols in the samples, which were expressed in μg/g oil.

The water contents in the oil samples were measured by Karl Fischer titrator (Titroline KF Trace, Schott Instruments, Mainz, Germany) (AOCS Ca 2e-84, 2009) (AOCS, 2009). Micellar sizes were analyzed with dynamic light scattering (DLS) using the Zetasizer Nano (Malvern Instruments, Worchestershire, U.K.). Light scattering was monitored for samples placed in vertical glass cuvettes of 10 mm width at a backscatter angle of 173° relative to the source, with an avalanche of photodiode detector at 25°C. The following values were used as approximates for sunflower and canola oils (i) viscosities 48.98 and 73 cP (Abramovic and Klofutar, 1998 and Kibbey *et al.*, 2014), (ii) refractive indices 1.475 and 1.471, and (iii) dielectric constants 3.79 and 3.06 (Ackman, 1983 and Semancik *et al.*, 2007), respectively for the two oils. All analyses were performed in duplicates.

3.3 Results and discussions

Due to the differences in fatty acid composition (**Table 3.1**) and the different oxidation temperatures (40°C for sunflower oil and 50°C for canola oil), these oils were expected to have different oxidation rates. The changes in fatty acid composition of the unpurified oils were monitored via the ratio of C18:1/C18:0, C18:2/C18:0 for both oils and C18:3/C18:0 for canola oil (Alireza *et al.*, 2010 and Marinova *et al.*, 2012). The most noticeable change was for C18:2/C18:0 for sunflower oil and for C18:1/C18:0 for canola oil, representing changes in the major unsaturated fatty acid in each oil (**Figure 3.1**).

Table 3.1. Fatty acid composition of sunflower and canola oils used in this study (relative percentage)

Fatty acids	Sunflower oil	Canola oil
Myristic, C14:0	0.3	n.d.
Palmitic, C16:0	9.3	6.5
Palmitoleic, C16:1	n.d.	0.3
Stearic, C18:0	4.7	1.8
Elaidic, C18:1n9t	0.5	1.2
Oleic, C18:1n9c	24.5	58.6
Linoleic, C18:2n6c	59.5	19.1
Arachidic, C20:0	n.d.	1.1
α-linolenic, C18:3n3	n.d.	9.3
Eicosenoic, C20:1n9	n.d.	1.2
Eicosadienoic acid, C20:2	0.5	0.4
Behenic, C22:0	0.6	0.3

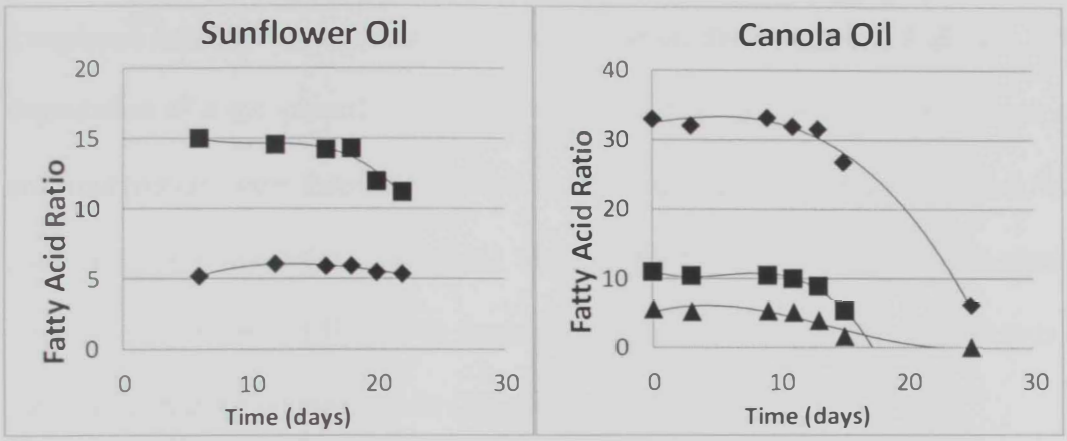


Figure 3.1. Changes in fatty acid ratios during the oxidation of unpurified sunflower oil (40°C) and canola oil (50°C) oil in 400 ml beakers; Symbols: ♦ is C18:1/C18:0, ■ is C18:2/C18:0, ▲ is C18:3/C18:0

The PV of unpurified sunflower oil in 400 and 800 ml beakers increased slowly from about 5 mEq/kg in the beginning to about 18 mEq/kg at the end of the induction period (IP) (day 16) (**Figure 3.2**). Unpurified canola oil, oxidized at 50°C, has an initial PV of about 2 mEq/kg, and its IP ended at day 9 where the PV was around 15 mEq/kg (**Figure 3.3**). The IP of these two oils are comparable with findings of (Viera and Regitano-d'Arce, 2001; Makhoul *et al.*, 2006; and Malcolmson and Vaisey-Genser,

2013). The PVs of purified oils were much higher, and their IP were shorter than the unpurified ones because purification removed tocopherols and other minor lipid components. The PV of purified sunflower oil increased slowly from 1 to 24 mEq/kg at day 6 in 400 ml beakers and to from 1 to 34 mEq/kg at day 5 in 800 ml beakers, where the IP ended. The results were in accordance with another study on sunflower oil stripped of tocopherols (Hras *et al.*, 2000). For purified canola oils, the oxidation reached the end of IP at day 2 and 1.5, with PV around 17.5 and 25 mEq/kg, in 400 and 800 ml beakers, respectively (**Figure 3.4**).

Initially, sunflower oil contained 600 mg/kg of α- tocopherol and canola oil contained α- and γ-tocopherol at 140 and 265 mg/kg, respectively. In both oils, the tocopherol content was reduced as oxidation proceeded (**Figure 3.2 & 3.3**). The degradation of α-tocopherol followed a second order of polynomial in sunflower oil and a polynomial order three in canola oil and the degradation of γ-tocopherol followed a second order polynomial in canola oil. The fact that tocopherols were not completely diminished at the end of IP may be explained by the presence of other antioxidants or surfactants in these unpurified oils (Ramadan, 2013).

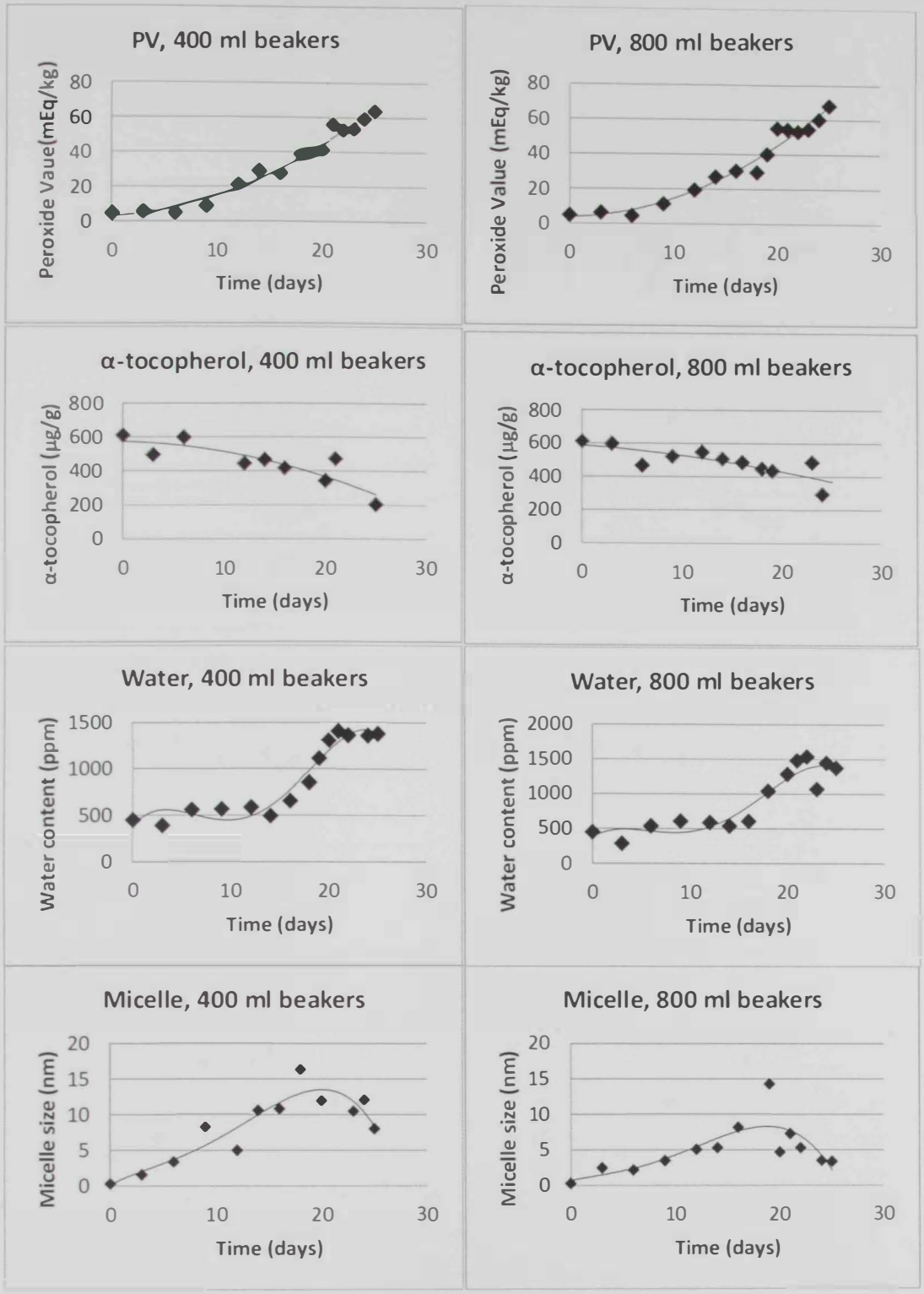


Figure 3.2. Degradation of α-tocopherol and evolution of hydroperoxides (measured as peroxide value), water content and micelle size in unpurified sunflower oil at 40°C in 400 and 800 ml beakers

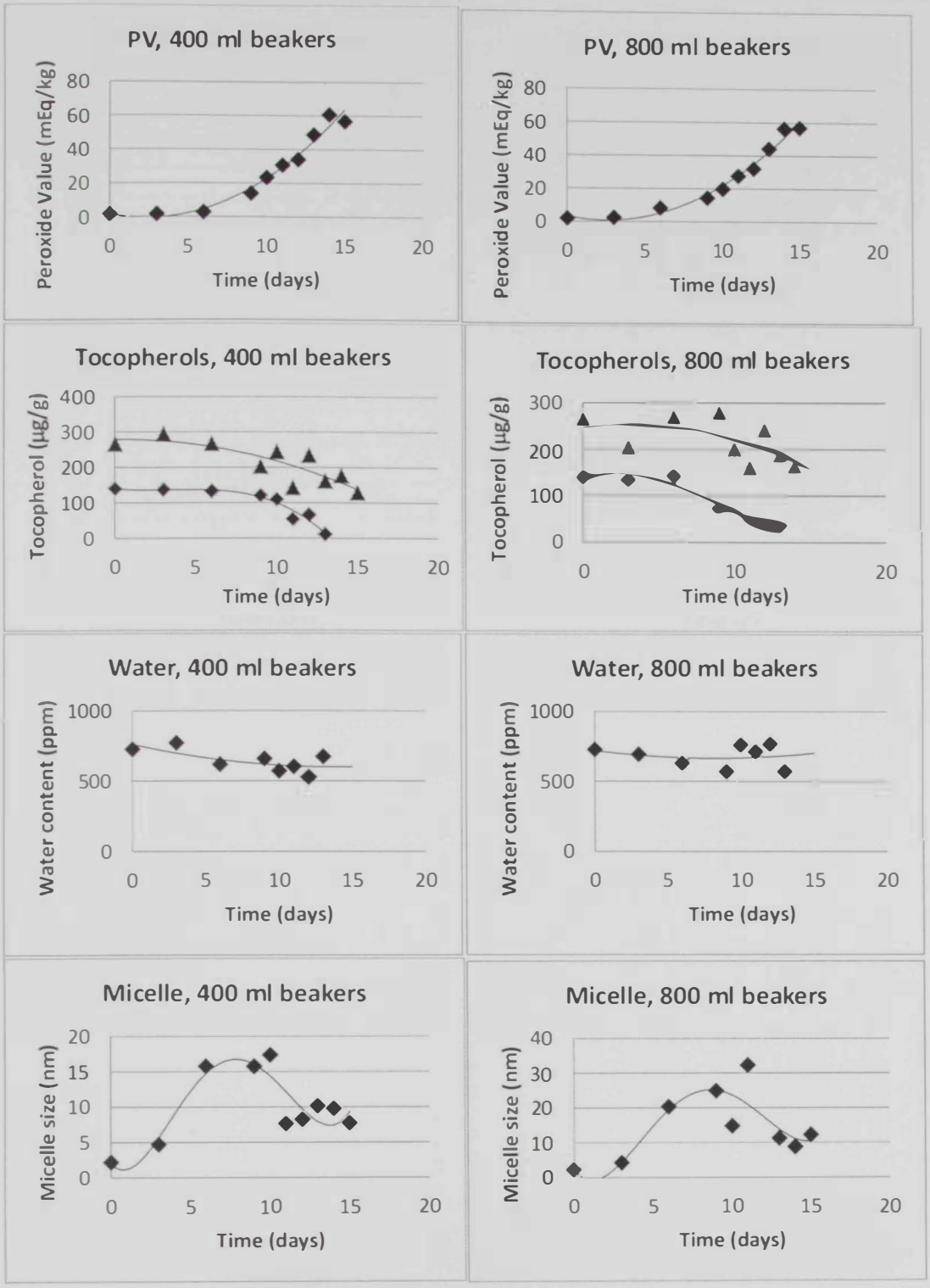


Figure 3.3. Degradation of α-tocopherol and evolution of hydroperoxides (measured a peroxide value), water content and micelle size in unpurified canola oil at 50°C in 400 and 800 ml beakers; Symbols of tocopherols: ♦ is α-tocopherol, ▲ is γ-tocopherol

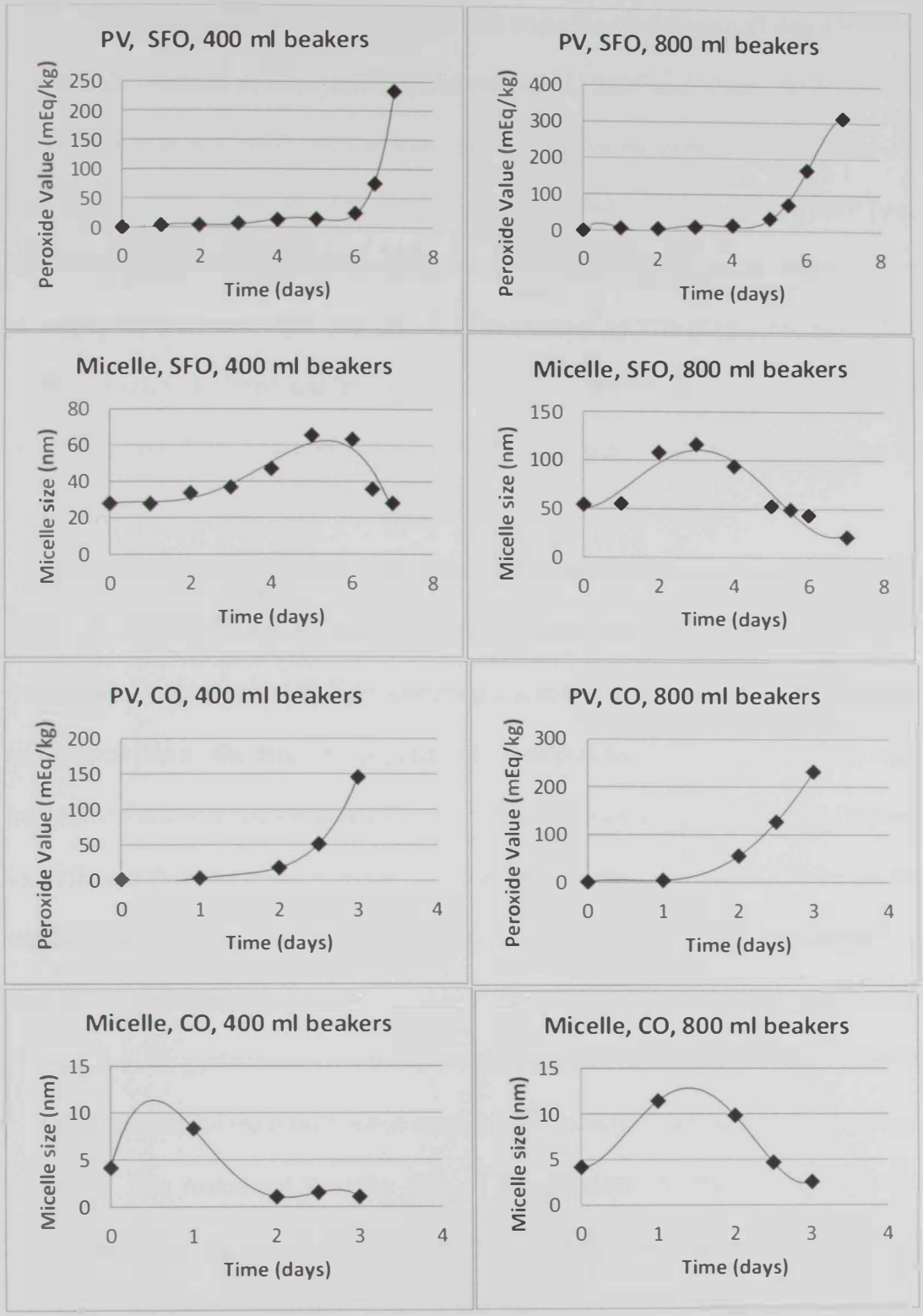


Figure 3.4. Evolution of hydroperoxides (measured as peroxide value) and micelle size in purified sunflower oil (SFO) at 40°C and canola oil (CO) at 50°C in 400 and 800 ml beakers

The water content (**Figure 3.2**) in fresh unpurified sunflower oil was 450 ppm, which is comparable to other results (Chaiyasit *et al.*, 2007 and Chen, 2012). During the oxidation of sunflower oil, the water content increased slowly until days 14-17, around the induction period, after which it increased sharply, up to 1300 – 1478 ppm. The initial water content in fresh unpurified canola oil was 728 ppm, the water content of canola oil increased until only day 6 to the values of 770-1113 ppm, but unlike sunflower oil, it declined and leveled off to around 530-770 ppm after the induction period (**Figure 3.3**). The reason for this difference may relate to the structures of hydroperoxides and the chemistry and products of their degradation.

In this study, an increase in micellar size was observed over time until the end of the IP. Micellar size was analyzed with dynamic light scattering (DLS), which determines the fluctuations in light scattering due to Brownian motions of the particles and relates this to the size of the particles. In unpurified sunflower oil, there were increases of micellar sizes (starting from ± 2 nm) until a certain period, day 18 (approx. 15 nm), and then the micellar sizes decreased drastically (**Figure 3.2**). Whereas the micellar size of unpurified canola oils grew from a size of ± 2.2 nm and reached its apex of ± 17 nm (day 10) and ± 32 nm (day 11) in 400 and 800 ml beakers, respectively (**Figure 3.3**). In purified sunflower oil, the micellar size grew gradually starting from ± 2 nm, reached its maximum value close to the induction period and then reduced gradually. The maximum micellar size of oil correlated to PV on day 5 (14.31 mEquiv./kg) and the end of the IP of the oils (day 6). The micellar size of purified canola oil reached its maximum on day 1 and the end of the IP of the oils was at day 2 for the 400 ml beakers and day 1.5 for the 800 ml beakers.

The increase in micelles size could be interpreted as amphiphilic molecules and hydroperoxides which begin to aggregate to form submicellar or pre-micellar

structures (Katre *et al.*, 2013). As the concentration of water and different hydroperoxides, the micelles grow in size and become well defined (Smit *et al.*, 1991; Pileni, 1997; Izquierdo *et al.*, 2002; Chaiyasit *et al.*, 2004 and 2007; Motshweni, 2007 and Katre *et al.*, 2013). When the concentration of micelles increases beyond a critical micelle concentration (CMC), these micelles start to have a lower stability due to decreased solubility in the oil and because of collisions and coalescence (Cason *et al.*, 2001). At the end of IP, when the concentration of hydroperoxides and other polar oxidation products increase, the large micelles collapse and smaller micelles are formed (Brimberg and Kamal-Eldin, 2003a and b; Garti, 2003; Momma *et al.*, 2004 and Laguerre *et al.*, 2011). These micelles influence the oxidation of bulk oils by acting as microenvironments or microreactors (Motshweni, 2007; Choi *et al.*, 2010 and Niazmand *et al.*, 2011). It was observed that the PV was not always in parallel with the micellar size, indicating there are other factors which influenced the size of micelles. For instance, the day when micellar size reached its maximum (in both unpurified oils), was slightly after the end of induction period of the oils. These dynamic, but thermodynamically stable micelles, can rearrange themselves, exchange water, surfactants and material contents and reform into two different micelles through fusion, interaction, collision, coalesce and flocculation (Pileni, 1997; Carvalho and Cabral, 2000; Pileni, 2006; and Motshweni, 2007).

CHAPTER FOUR

Stabilization of cod liver oils with quaternary antioxidants (α-tocopherol, ascorbyl palmitate, phosphatidylcholine and L-lysine)

4.1 Introduction

The health benefits of omega-3 fatty acids, which are commonly found in fish oils, have been well addressed. These benefits include antiartherosclerotic, antithrombotic, antiarrhythmic, anti-inflammatory activities (Taherian *et al.*, 2011) and the inhibition of cancer by induction of tumor cell apaptosis (Kathirvel and Rupasinghe, 2011). The World Health Organization (WHO) has recommended the consumption of an equivalent of 200 - 500 mg of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) per week (Kathirvel and Rupasinghe, 2011) and as a result, many food products are being fortified with fish oils (Horn *et al.*, 2009).

Because of the high content of omega-3 fatty acids, fish oils are highly susceptible to oxidation (Kulas *et al.*, 2002). Attempts to protect omega-3 oil against oxidation by the combinations of natural antioxidants have been practiced (Kamal-Eldin and Yanishlieva, 2002). Ternary antioxidant systems (tocopherols, ascorbyl palmitate and lecithin or with another synergist) revealed that there are synergisms among the antioxidants which have been reported to be a success in protecting fish oil and omega-3 rich oils against oxidation. A study by Han *et al.*, (1991) in sardine oil at 30°C, found that the combination of δ-tocopherol (4000 μg/g), ascorbic acid (200 μg/g) and lecithin (3000 μg/g) inhibited oxidation of the oils, measured by primary and secondary oxidation products. Drusch and colleagues (2008) found that α-tocopherol (100 μg/g) with δ-tocopherol (1000 ppm), ascorbyl palmitate (500 μg/g) and lecithin (2000 μg/g) resulted in the most efficient hydroperoxides (LOOH) reduction, but not the propanal content in the autoxidation of stripped refined fish oil at 20°C. Serfert *et al.*, 2009 found that the combination of α – and δ-tocopherol (100 – 1000 μg/g), ascorbyl palmitate (500 μg/g) and lecithin (1115 μg/g) inhibited lipid oxidation in the refined fish oil at 20°C, with the most notable effects being achieved by δ-tocopherol.

However the quaternary combinations of antioxidants in stabilizing fish oil, to the best of our knowledge has not yet been studied, and is investigated in this study. Another additive used in this study is L-lysine. Synergy between L-lysine and other additives have been reported (Hidalgo *et al.*, 2006 and 2009). If combinations of additives can be applied each at lower concentration to oils, these would potentially give commercial benefits and increase the effectiveness of antioxidants in protecting bulk fish oil. The additives employed in this study are α-tocopherol (α-TOH) as a primary antioxidant and three synergists: ascorbyl palmitate (AP), L-lysine and phosphatidylcholine (PC). The aims of this study were therefore to investigate the effects of combinations of these four additives (α-TOH, AP, L-lysine and PC) by response surface methodology (RSM), in protecting cod liver oil against autooxidation. Oxidation products: conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) and α-TOH content are measured during the study.

4.2 Materials and methods

Fish oil and additives

Cod liver oil (Sigma Aldrich, St. Louis, USA) used in this study had the following fatty acid composition: C14:1 (2.4%); C15:0 (0.4%); C15:1 (0.5%); C16:0 (16.7%); C16:1 (0.6%); C17:0 (2.6%); C17:1 (1.7%); C18:0 (3.5%); C18:1n9c (22.2%); C18:2n6t (0.6%); C18:2n6c (2.9%); C18:3n6 (0.3%); C18:3n3 (0.9%); C20:1n9 (12.4%); C21:0 (2.8%); C20:4n6 and C22:1n9 (10.1%); C23:0 (0.9%); C20:5n3 (8.7%); C24:1n9 (1.0%) and C22:6n3 (8.9%). The oil was kept in a closed amber bottle and refrigerated until the day of use. The oil was found to contain 1100 μg/g α-tocopherol and 0.02321 mmol conjugated dienes/g, at the time of use.

The effects of four additives (α-tocopherol, ascorbyl palmitate, phosphatidylcholine and L-lysine) on the stabilization of the cod liver oil were studied

using the statistical design explained in **Table 4.1**. Additives were dissolved in solvents as follows: α-tocopherol in hexane, ascorbyl palmitate in hexane : ethanol (2 : 3 (v/v)), L-lysine and phosphatidylcholine in methanol. Solution of L-lysine in methanol was heated for a few minutes in a water bath in order to assist the solubility. After the additives were placed in respective tubes, they were mixed carefully for a few seconds before the solvents were removed under a stream of nitrogen. The oil (2 g) was then added to the tube and was mixed for a few seconds. Three sets of the 27 design treatments and 3 control tubes (untreated cod liver oil) were used for incubation in order to minimize the effects of the height of oils taken each day. All tubes were then placed in an incubator at 30°C.

Table 4.1. Response surface design of experiments testing the effects of four additives (independent factors) on the stability of cod liver oil at 30°C

Design treatment	Independent factors (μg/g oil)				Responding variables		
	(X1) α-TOH*	(X2) AP	(X3) PC	(X4) L-lysine	Slope of CD*	Slope of TBARS*	Slope of loss of α-TOH*
1	500	100	1000	5000	0.029	8.268	-254.1
2	500	500	1000	1000	0.090	4.287	-118.1
3	500	100	5000	1000	0.054	4.732	-32.4
4	500	500	1000	9000	0.064	7.045	-149.7
5	100	900	5000	5000	0.021	5.872	-39.3
6	900	500	9000	5000	0.004	5.396	-204.0
7	500	500	5000	5000	0.030	5.984	-88.8
8	500	500	5000	5000	0.071	3.962	-114.2
9	500	900	5000	1000	0.038	5.157	-187.6
10	100	500	9000	5000	0.090	1.306	-230.4
11	900	100	5000	5000	0.030	5.137	-102.2
12	900	500	5000	9000	0.068	2.762	-67.9
13	100	500	5000	1000	0.003	2.510	-218.2
14	500	900	5000	9000	0.050	2.867	-223.8
15	900	500	5000	1000	0.015	4.347	-171.9
16	900	900	5000	5000	0.007	3.616	-121.3
17	500	100	5000	9000	0.019	1.863	-169.4
18	100	500	5000	9000	0.036	2.161	-105.7
19	100	500	1000	5000	0.077	3.665	-135.9
20	500	100	9000	5000	0.055	1.489	-144.6
21	900	500	1000	5000	0.048	5.020	-109.9
22	500	500	9000	1000	0.288	2.063	-125.4
23	100	100	5000	5000	0.031	1.995	-50.0
24	500	500	5000	5000	0.061	5.809	-139.8
25	500	500	9000	9000	0.108	1.730	-173.3
26	500	900	1000	5000	0.165	5.154	-194.2
27	500	900	9000	5000	0.009	8.102	-193.4

α-TOH is α-tocopherol, AP is ascorbyl palmitate, PC is phosphatidylcholine, CD is conjugated dienes, TBARS is thiobarbituric acid reactive substances.

*The original oil already contained 1100 μg/g α-TOH.

*Slopes of CD, TBARS and loss α-TOH were determined between day 0 and day 4, and are given in units of mmol g^{-1} day^{-1}, mg g^{-1} day^{-1} and μg g^{-1} day^{-1}, respectively.

Oxidation measurements

Each day (between day 0 and day 7), one set of tubes was analyzed for their contents of the three response variables: conjugated dienes (CD) according to Medina *et al.* (2010), thiobarbituric acid reactive substances (TBARS) according to Hamam and Shahidi (2005) and α-tocopherol (α-TOH) according to Kayden *et al.* (1973).

Statistical analysis

Response surface methodology (RSM) with Box-Behnken design, using Minitab 17 statistical software (Minitab Ltd, Coventry, UK), was used to design the experiments, evaluate the effects of four additives (independent factors) on lipid oxidation of cod liver oil and generate response surfaces. Each factor was tested at three levels (-1, 0 and +1). A total of 27 design treatments were tested, as shown in **Table 4.1**, and the results were used by the software to generate models describing the effects of the 4 independent factors (additives) (α-TOH, AP, PC and L-lysine) on the 3 response variables (CD, TBARS, loss α-TOH). Based on analysis of variance (ANOVA), a statistical significance is relied on a probability value of $p<0.05$. The goodness of model fitting was considered based on coefficient of determination (R^2), which indicates the proportion of the variation in response variable that is explained by the independent variable. Contour plots were constructed to depict the interaction between α-TOH and each of the three other independent variables, and was also used to detect the optimum regions of the preferred response. Model adequacy or fitness was tested using normal probability plots. Optimum levels of the additives were obtained from the response optimizer menu from the software.

4.3 Results and discussions

Oxidation rates of control and treated cod liver oils

The control (untreated) sample had an induction period (IP) of four days, in agreement with Medina *et al.*, (2010). The differences between the control and treated samples with regard to IP are not large. According to Yanishlieva and Marinova (1992), antioxidant effects are not only reflected by the length of the IP, but also by the decreased of reaction rate. The slopes of the formation of CD and TBARS and loss of α-TOH of the control sample are 0.0054 mmol g^{-1} day^{-1}, 6.760 mg g^{-1} day^{-1} and -158.5 μg g^{-1} day^{-1}, respectively. Based on these, the best treatments compared to control were treatments number 3, 5, 7, 12 and 23 (**Table 4.1**). The slope of the CD of the control was lower than most of the treatments. This may be because these samples contained a high level of α-TOH which increased the rate of oxidation during IP (Kulas and Ackman, 2001). The oxidation kinetic curves of the control and treated samples oxidized at 30°C are presented in **Figure 4.1**.

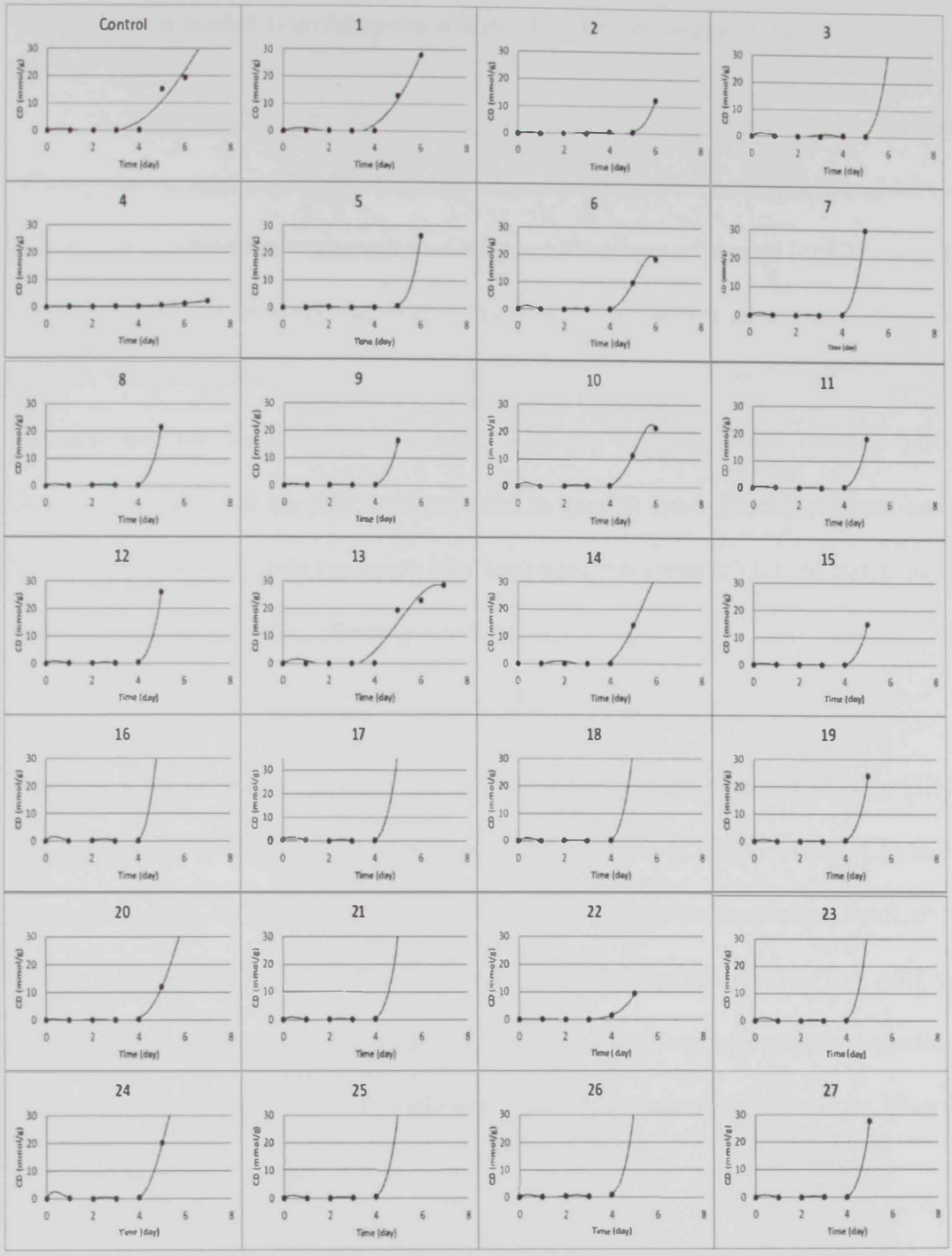


Figure 4.1. The evolution of conjugated dienes (CD) during the autoxidation of cod liver oil at 30°C. For treatments, see **Table 4.1**.

The statistical model describing the effects of additives on the stability of cod liver oils

A study by Yanishlieva and Marinova (1992) suggested that antioxidant efficacy can be measured by the rate oxidation during the IP. The protective effects of antioxidant combination were evaluated based on the slopes of change for CD, TBARS and loss of α-TOH between day 0 and day 4. The data was used to build statistical models, which explores the relationships between the four additives (independent variable) and the three response variables (slopes of CD, TBARS and α-TOH). Quadratic polynomial models were selected to predict the response surface model based on the β coefficients (calculated by least square regression) for the independent variables according to the following equation:

$$Y = \beta 0 + \sum_{i=1}^{k} \beta iXi + \sum_{i=1}^{k} \beta iiXiXi + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta ijXiXj + \varepsilon$$

Where Y is the predicted response variable (slopes of CD, TBARS or loss of α-TOH), k is the number of factors, i = factor 1 to 4, j = factor $i + 1$, β_0 is a constant, βi is the linear coefficient, β_{ii} is the squared coefficient, β_{ij} is the interaction product coefficient, ε is the random error variable. The four independent factors are α-TOH (X_1), AP (X_2), PC (X_3) and L-lysine (X_4). The models (**Table 4.2**) contain three linear, three quadratic and three interaction terms. Coefficients from the coded variables are directly presented in the equations.

Table 4.2. Equations describing the responses (Y) to the coded levels of additives*

Response (Y)	Model equation	R^2	p-value
Slope of CD	$Y = 0.0539 - 0.00710\ X_1 + 0.00589\ X_2 - 0.01624\ X_3 + 0.01113\ X_4 - 0.0202\ X_1\ X_1 - 0.0103\ X_2\ X_2 + 0.0201\ X_3\ X_3 - 0.0042\ X_4\ X_4 - 0.0033\ X_1\ X_2 - 0.0144\ X_1\ X_3 + 0.0049\ X_1\ X_4 - 0.0457\ X_2\ X_3 + 0.0115\ X_2\ X_4 + 0.0302\ X_3\ X_4$	0.7268	0.112
Slope of TBARS	$Y = 5.252 + 0.731\ X_1 + 0.607\ X_2 - 1.113\ X_3 - 0.389\ X_4 - 1.175\ X_1\ X_1 + 0.133\ X_2\ X_2 + 0.042\ X_3\ X_3 - 1.458\ X_4\ X_4 - 1.349\ X_1\ X_2 + 0.684\ X_1\ X_3 - 0.309\ X_1\ X_4 + 2.432\ X_2\ X_3 + 0.145\ X_2\ X_4 - 0.773\ X_3\ X_4$	0.8187	0.012
Slope of loss of α-TOH	$Y = - 114.3 - 47.2\ X_1 - 17.2\ X_2 + 12.0\ X_3 - 29.3\ X_4 + 56.7\ X_1\ X_1 - 33.8\ X_2\ X_2 - 42.3\ X_3\ X_3 + 1.8\ X_4\ X_4 - 7.5\ X_1\ X_2 - 63.1\ X_1\ X_3 + 76.8\ X_1\ X_4 - 27.2\ X_2\ X_3 + 25.2\ X_2\ X_4 - 4.1\ X_3\ X_4$	0.7959	0.054

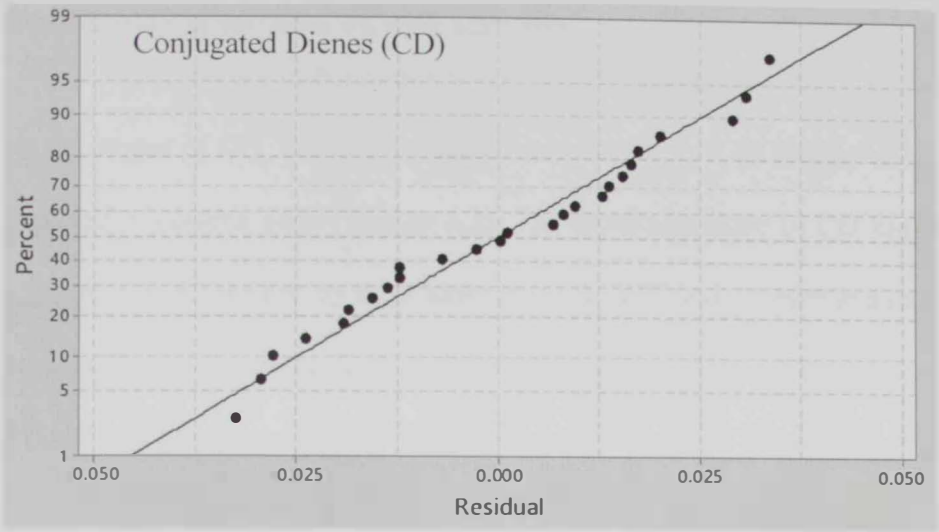
*Y is the predicted slope of formation of conjugated dienes (CD), thiobarbituric acid reactive substance (TBARS) or loss of α-tocopherol (α-TOH), X_1 is the coded level of α-TOH, X_2 is the coded level of AP, X_3 is the coded level of PC and X_4 is the coded level of L-lysine (see **Table 4.1**).

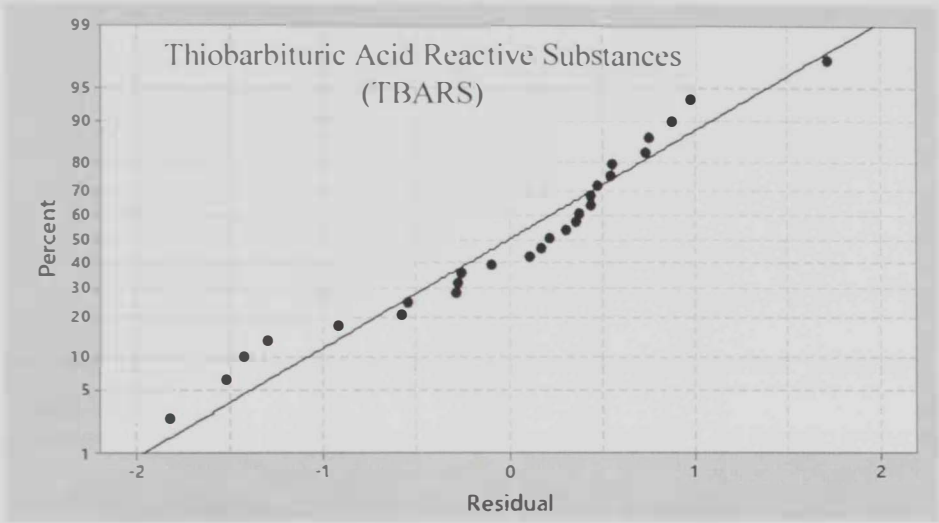
While evaluating the response of CD (slope between day 0 and day 4), one sample caused a high variation in the statistical analysis and was removed from the data set. The slope of CD formation (between day 0 and 4) was found to fit a second order polynomial model with $R^2 = 0.7268$ and p-value = 0.112. However this was not accepted as a good model although similarly weak models have been accepted by others (Can and Ersan, 2013 and Pankyamma *et al.*, 2014). Regarding the response of the slope of TBARS formation (between day 0 and 4), it was found to fit a second order polynomial model with $R^2 = 0.8187$, p-value = 0.012 and with insignificant lack-of-fit (p=0.519). Concerning the response of α-TOH, two samples caused high statistical variation and were removed from the data set. Based on this, the slope of loss of α-TOH (between day 0 and 4) was also found to fit to a second order polynomial

or full-quadratic model with R^2 = 0.7959, p-value close to 0.05 and p-lack of fit of 0.290 (insignificant lack-of-fit).

The best parameter to evaluate the protective effect of the four additives in cod liver oil was TBARS. The fact that the model for CD is weak may be justified by peroxide value (PV) which is considered a less satisfactory measurement of oxidation in lipids containing fatty acids with 3 or more double bonds, due to prospective side reactions (such as the breakdown of hydroperoxide to volatile compounds, which secondary products cause fish oils to be unacceptable in terms of sensory) (Frankel *et al.*, 2002). Since significant amount of CD and TBARS are only generated from oxidation of PUFA and fish oils, therefore measuring them were suggested for lipids of these kinds (Elisia *et al.*, 2013 and Poyato *et al.*, 2013).

Diagnostics of the test for the residuals of fitted are given by the normal probability plots for residuals (**Figure 4.2**). The figures show that residuals are normal which proves model adequacy.





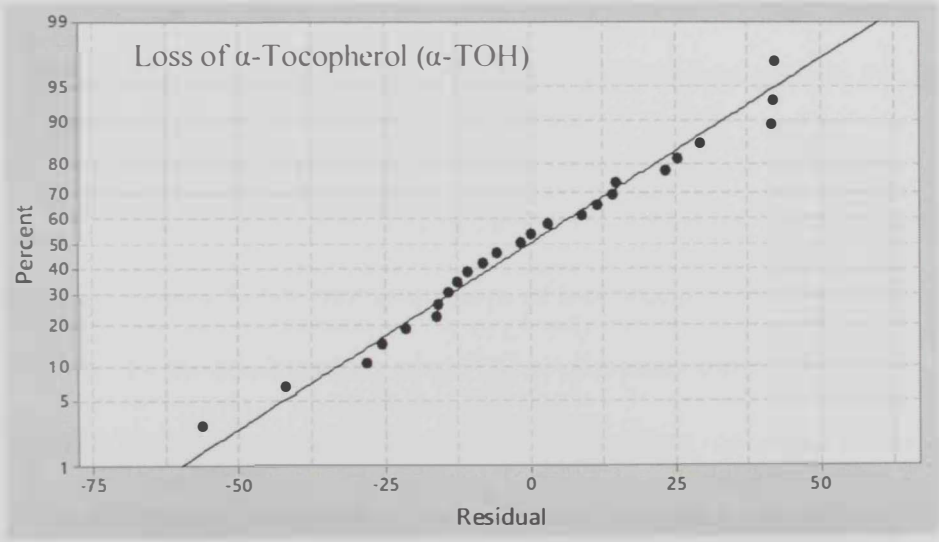


Figure 4.2. Normal probability plot for residual of slope of formation of CD and TBARS and loss of α-TOH

Stabilization of cod liver oil with additives

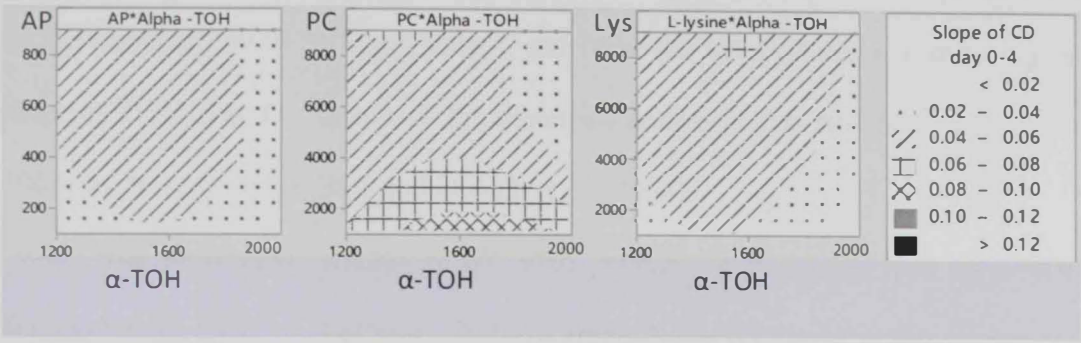
The influence of the interaction of α-TOH and each of the other three additives on the slopes of CD, TBARS formation and loss of α-TOH formation (day 0 to 4) is depicted in contour plots (**Figure 4.3**). The minimum slope of CD formation during day 0 to 4, was obtained by the interaction of α-TOH and AP both at a minimum level, or α-TOH at a maximum level and AP at all ranges. Also the minimum slope of CD formation was attained with α-TOH at maximum level and PC at a middle to maximum level. The minimum slope of CD formation can also be achieved when α-TOH and L-lysine both at minimum levels or α-TOH at maximum levels together with L-lysine from a minimum to a middle level. This analysis of the slope of CD will not be considered (as p-value of regression is more than 0.05) and the main focuses will be on TBARS and loss of α-TOH.

The minimum slope of TBARS formation during day 0 to 4 (**Figure 4.3**) was attained by a combination of α-TOH at levels close to the minimum with AP at midpoint, or α-TOH at minimum levels together with PC at middle to maximum levels, or α-TOH and L-lysine both at minimum or both at maximum levels, or α-TOH at minimum together with L-lysine at all ranges. From these combinations, significant interactions were found for α-TOH and AP, and between AP with PC, with p-values of 0.050 and 0.002, respectively.

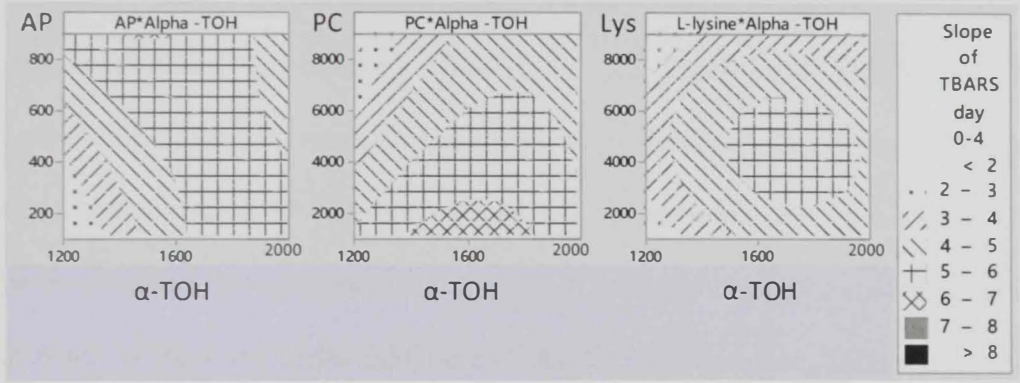
Contour maps of the slope of tocopherol during day 0 to 4 showed saddle point trends, **Figure 4.3**. The minimum slope of tocopherol reduction from day 0 to 4 was achieved by the combinations of α-TOH at minimum and AP at all ranges, or α-TOH at levels higher than the minimum point but less than maximum levels together with AP at all ranges. The minimum slope of tocopherol loss was also attained with α-TOH at minimum and PC at mid to maximum, or α-TOH middle level with PC at all ranges, or α-TOH at maximum level together with PC at minimum to middle level. The

minimum slope of tocopherol reduction could also be achieved with α-TOH at minimum and L-lysine around middle point, or α-TOH at mid to high levels together with L-lysine at a high level. From the above combinations, however only interactions of α-TOH and L-lysine, and α-TOH with PC were significant with p values < 0.05.

Conjugated Dienes (CD)



Thiobarbituric Acid Reactive Substances (TBARS)



α-Tocopherol (α-TOH)

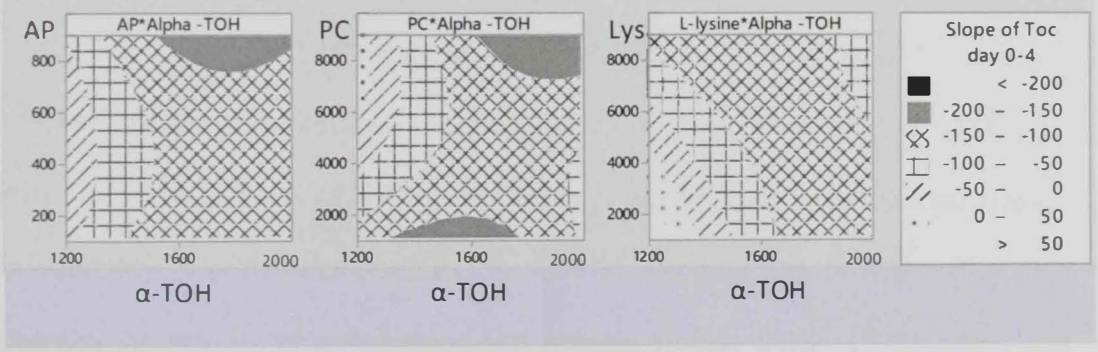


Figure 4.3. Contour maps of the interaction effect of α-tocopherol and each of the other three additives and the slope of CD, TBARS and α-TOH between day 0 and day 4

Thus, it can be concluded that α-TOH at levels close to minimum 1200 μg/g were effective. It has been reported in some studies that at a high level, α-TOH causes prooxidative effects or loss of efficacy effects in fish oils at different levels. In salmon and mackerel oils and menhaden triacylglycerols, the loss of efficacy was at more than 100 ppm (Kulas and Ackman, 2001 and Belhaj *et al.*, 2010) and more than 200 ppm (Zuta *et al.*, 2007), due to potential side reactions (Let *et al.*, 2007). AP concentrations in the range of 100 to 850 μg/g were found to be the most effective in reducing the slope of TBARS and residual α-TOH, which are comparable to results reported in the literature (Carelli *et al.*, 2005 and Drusch *et al.*, 2008).

The synergistic effects of AP with primary antioxidants such as α-TOH (Frankel *et al.*, 2002 and Let *et al.*, 2007), were probably due to AP ability to partition in the water phase (Frankel *et al.*, 2002), reduce tocopheroxy radicals to tocopherols (regeneration of tocopherols) (Serfert *et al.*, 2009) and reduce *cis, trans*-hydroperoxides to their related hydroxyl forms (Serfert *et al.*, 2009). Our study showed that a higher level of AP did not result in a better protection to the fish oil. Some studies have advocated that at certain (high) levels, AP may have a prooxidative effect in the presence of transition metal ions (i.e. Fe^{3+} and Cu^{2+}) (Horn *et al.*, 2009 and Jayasinghe *et al.*, 2013).

PC was found to be the most protective at the level of 5000 μg/g. The effective PC level reported in the bulk sardine and salmon oils varied from 1000 to 10000 μg/g (Han *et al.*, 1991; King *et al.*, 1992a and Serfert *et al.*, 2009). PC was reported to assist the solubility of primary antioxidants in bulk oil and emulsion system, which thus enhances the activity of antioxidants and reduces surface tensions (Azizkhani and Zandi, 2010; Zou and Akoh, 2013). In addition, PC or lecithin plays a physical role by forming an interfacial molecular complex at the lipid/water interface (Klinkesorn and

McClements, 2009; Belhaj *et al.*, 2010; Sorensen *et al.*, 2011 and Zou and Akoh, 2013). It is also thought that PC (or lecithin) may have a metal chelating and radical scavenging activity (Drusch *et al.*, 2008) while Salminen *et al.*, (2013) asserted that phospholipids contribute antioxidant effects by "unknown mechanism(s)".

Regarding the level of effectiveness of L-lysine in the oils, it is effective in the range 1000 - 5000 μg/g. Effective L-lysine levels reported in literature varied from 200 - 10000 μg/g, depending on the kinds of oils substrates, temperature used in the studies and the presence of other additives (Ahmad *et al.*, 1983b; Hidalgo *et al.*, 2005 and 2009). Some amino acids (including L-lysine) were reported to exhibit synergisms by covalently binding to Trolox-C, generating Troloxyl-amino acids with higher antioxidant activities (Naguib, 2000). According to Farvin *et al.*, (2014), the shielding effect of some amino acids by allocating at water/oil interface, contributes to the amino acid antioxidant activity. The results from this study showed that lower and middle levels of L-lysine gave a better protection to the oils, rather than at a higher level.

Synergisms and recommended combination of additives

Synergism between the four additives was considered based on the slope of formation of TBARS and loss of α-TOH. Based on the least rate of change of the slope of TBARS and loss of α-TOH (day 0 to 4), the recommended best levels of combinations of additives are α-TOH (1200 μg/g), AP (100 μg/g), PC (9000 μg/g) and L-lysine (1000 μg/g).

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

A comprehensive literature review was performed to systematically describe the physical effects of antioxidants and synergists on lipid oxidation during the induction period (**Chapter 2**). Antioxidants and minor components are surface active and form association colloids or supra molecular structures in a continuous lipid phase such as bulk oils. Small amounts of water reside in the micellar core, while antioxidants amphiphilic minor components (e.g. phospholipids, monoacylglycerols and free fatty acids) act as surfactants and affect the stability of the microenvironments in bulk oils. These amphiphiles are capable of changing the locations of pro- and antioxidants in the microenvironments (which are the active catalytic and sites of oxidation) thereby accelerating or decelerating the lipid oxidation rate. The structure of triacylglycerols and other lipid-soluble molecules affect their organization and hence, oxidation in bulk oils. The location of antioxidants in the micelles can vary, generally antioxidant head groups have more affinities and are located at the water/oil interfaces, and thus scavenge radicals according to their locations and concentrations in the micelles. Based on this scenario, besides acting as free-radical scavengers via hydrogen donation, antioxidants protect oils by the physical stabilization of the micelles. There is a cut-off effect (optimum value) of the level of antioxidants that can protect the oil, depending on their hydrophilic/lipophilic balance and concentrations. Synergists modify physically the microenvironment, thus influencing the oxidation. Paradoxical outcomes which could not be explained previously, indicated the complexity of the antioxidant effects. This is now partly elucidated by understanding the supramolecular chemistry of lipid oxidation and antioxidants.

The aim of the research in **Chapter 3** was to follow the formation of water and evolution of micellar size during the oxidation of sunflower and canola oils in purified and unpurified forms. In all oils, the water content and micellar size increased as the

peroxide value (PV) increased until the end of the induction period (IP) then micellar size dropped. After the IP, water content continued to increase in unpurified sunflower oil, but not in unpurified canola oil, which could be attributed to the different pattern of degradation of hydroperoxides from the latter. The evidence that the increases of water content and micellar size coincided with the end of the IP, supports the theory that micelles are the sites where oxidation is dominant in bulk oils.

In **Chapter 4**, the combination of quaternary antioxidants consisting of α-tocopherol (α-TOH), ascorbyl palmitate (AP), phosphatidylcholine (PC) and L-lysine was found to protect cod liver oil against autoxidation at 30°C. The study used response surface methodology (RSM) to find that the additives can reduce the rates of formation of conjugated dienes (CD) and thiobarbituric acid reactive substance (TBARS) and loss of α-tocopherol (α-TOH). Mathematical models following second order polynomial with good confidence ($p \leq 0.05$) were obtained for the slopes of TBARS and α-TOH. Normal probability plots of the parameters confirmed models adequacy. The recommended optimum levels of the additives (in μg/g) to protect cod liver oils from autoxidation at 30°C are α-TOH at 1200, AP at 100, PC at 9000 and L-lysine at 1000 μg/g. Higher levels of α-TOH and AP did not give better protection to the oils or caused a loss of antioxidant efficacy, as opposed to when the additives were applied at lower levels.

Finally, the understanding of both chemical and physical aspects of bulk oils will contribute to the development of the knowledge of lipid oxidation and help to protect lipid products in a more integrated manner. This approach particularly will be useful in the research and processes where PUFAs are extensively used in the products, such as in healthy, functional food products.

The results from this study support the following recommendations for research that can be done in the future to develop a better understanding of the physical and chemical aspects of bulk oils oxidation and how they relate to each other, e.g.:

1. To understand and access and use technologies, and additional methodologies to examine the physical effects of lipid oxidation, such as using small/wide angle x-ray scattering (SAXS/WAXS).
2. The time when the maximum micellar size is reached does not always coincide with the time when the IP ends. This may suggest that there are (chemical) factors which influence the physical aspects. Further investigations of these factors may be interesting.
3. To create physical entities using additives and antioxidants, and study the characteristics of these entities.
4. To investigate the entities and changes in bulk oils when additives are not acting as synergists or become prooxidants.

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